BIOCHEMICAL SOCIETY SYMPOSIA
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A Symposium held at
London School of Hygiene and Tropical Medicine
on 30 October 1948

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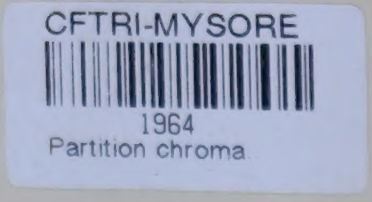
CAMBRIDGE
AT THE UNIVERSITY PRESS
1951

PUBLISHED BY
THE SYNDICS OF THE CAMBRIDGE UNIVERSITY PRESS

London Office: Bentley House, N.W.1
American Branch: New York

Agents for Canada, India, and Pakistan: Macmillan

First printed 1949
Reprinted „
„ *August* 1950
„ *January* 1951



Printed in Great Britain at the University Press, Cambridge
(Brooke Crutchley, University Printer)

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1. INTRODUCTION

By E. BALDWIN

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'The invention of a new, specialized laboratory procedure brings about rapid conquests in new fields of science and technology; finally, it exhausts itself and is replaced by a still more practical method. The method of chromatographic adsorption, invented by the talented Russian botanist, Prof. M. Tswett, makes possible spatial separation of the components of a mixture. It is just now at the beginning of a brilliant development; it offers a simple experimental procedure to the investigator, especially in the field of both pure and applied organic chemistry, of biochemistry and of physiology.' Thus wrote L. Zeichmeister in the preface to the first edition of his important *Principles and Practice of Chromatography* (see Zeichmeister & Cholnoky, 1938).

The separation of chemical substances by chromatographic adsorption is in many cases preceded by partial fractionation of the original mixture by partition between immiscible solvents. Adsorption chromatography has now passed into common usage for innumerable chemical and biochemical purposes, and has been supplemented by the newer technique of partition chromatography, a linear descendant of the original Tswett technique, which forms the subject of the present Symposium. Since its introduction by Martin & Synge (1941) and more recent elaboration by Consden, Gordon & Martin (1944), this new method has invaded the vast majority of chemical and biochemical laboratories in this country and elsewhere. It has invaded, too, almost every field of biochemical research. The present Symposium includes discussions of applications of the method to studies of the chemistry and metabolism of amino-acids, proteins, carbohydrates and fats, as well as to the separation of purines and pyrimidines and some large and important groups of natural pigments. These represent only some of the more important applications, for few methods, probably, have developed so rapidly and in so many diverse directions. And, as the contents of the present volume make clear, the method is still far from exhausting itself. Indeed, new applications, new ideas and new technical devices are still being introduced.

In introducing this Symposium perhaps it is not out of place to point to a few of the reasons that have made the method of partition chromatography so important. Much of the total time expended on biochemical research is devoted, in one way or another, to studies of the relationship

between chemical structure and biological activity. So intimate and so profound is this relationship that many cases are known in which a slight change in its structure—a change which from a purely chemical standpoint might seem insignificant—will totally destroy the characteristic biological activity of a given chemical compound. Perhaps these facts seem less surprising nowadays than they did a decade or two ago, for much has been learned about enzymes and enzymic specificity in the interval. But the structure/activity relationship is nevertheless still of profound and even vital importance in many fields of biochemistry, pharmacology and physiology.

Substances of similar structure, while often differing fundamentally in biological activity, tend to exhibit similar chemical properties. Much of the biochemist's time and energy have, in the past, been necessarily spent in attempts to isolate, often from mixtures containing closely related compounds, some substance of biochemical importance. The classical methods of organic chemistry—fractional distillation, fractional crystallization and the like—have yielded many notable successes in the past, but are often extremely laborious and commonly involve the loss of considerable proportions of the starting material which, when it is biological in origin, is not infrequently in short supply. But comparatively small differences of adsorbability, or of solubility in pairs of immiscible solvents, frequently suffice to allow of separation, purification, estimation, recovery and isolation of one substance from a complex mixture by suitable chromatographic procedures. By an appropriate choice of adsorbents and solvents, and by modification of the quantities of these materials, it is usually possible to work with anything from a fraction of a milligramme upwards, so that chromatographic methods are valuable not only to the research worker who first isolates a few milligrammes of some new biochemical or pharmacological principle but also to the worker who studies its chemistry and metabolism on the larger scale, and even to the manufacturing chemist who eventually puts the product on the market. Small wonder, then, that chromatography has found a warm welcome in our hearts.

Several problems confront the worker who attempts to use the partition technique for a new purpose. In his choice of solvents he may find guidance in the partition coefficients of his compounds if these happen already to be known. In his search for a supporting medium for the stationary phase of his system he will probably try silica gels, starch or cellulose first of all, or filter paper if he proposes to operate on the very small scale. There then remains the development of suitable procedures for the detection and estimation of the substance or substances under investigation, and here classical methods can usually be put to service. Recourse may be had to measurements of pH, to titration,

to spectrophotometry, to refractometry, fluorimetry or what not. But beyond this it is still up to the investigator himself to carry out preliminary trials, often in large numbers, for the theoretical background of partition chromatography, like that of its adsorption counterpart, is still too rudimentary to allow of much in the way of accurate prediction. The development of new partition methods is still essentially a problem to be tackled at the bench rather than in the library.

I therefore welcome particularly the first contribution to this Symposium. It will, I know, be valuable to all of us to have, collected together and discussed, the general principles and the theoretical background on which we may hope in due time to build. Ultimately, as we gain knowledge and understanding, we may hope to be able to choose, on purely theoretical grounds, a system appropriate for the performance of any given task and thus to avoid the preliminary phases of trial and error which at present consume so much time and labour.

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2. SOME THEORETICAL ASPECTS OF PARTITION CHROMATOGRAPHY

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It is intended in this contribution to interpret rather widely the theoretical aspects and to begin with a disquisition on the problems of purification in general, in order that partition chromatography may be seen in a framework of related methods, so that possible variants, not hitherto explored, may be easier to discern.

There are two fundamental problems in purification: (*a*) the separation of substances originally present in different phases and (*b*) the separation of substances present in the same phase.

(*a*) Many physical properties are employed in the separation of already pre-existing phases: size, shape, hardness, density, adhesiveness, electrical and magnetic behaviour. In general, in the separation of solid phases from one another, great purity is difficult to achieve, and chemical methods, changing the nature of some of the phases, have to be employed.

(*b*) Where substances are present in the same phase there are two types of separation possible, separation by distributing between different phases and separation by diffusional transport. The point does not have to be laboured that all 'chemical' methods of separation are means of changing the physical properties of the substances involved so that one or both of these basic methods may be used to greatest advantage. In diffusional processes the components move differentially under pressure, concentration or temperature gradients, or in centrifugal, electric or magnetic fields. It is characteristic of diffusional processes that the degree of enrichment achieved is dependent on the magnitude of the gradient, or the distances the components move in the fields. Some diffusional processes use membranes, whose function may range from preventing unwanted movements of fluids, when the membrane is equally permeable to all components, to acting as microsieves by manifesting permeability, maintaining in effect distinct phases more or less in equilibrium with each other, which but for the membrane would be miscible. The behaviour of such diffusional systems is more characteristic of the phase-change methods discussed below. But however the relative enrichment has been achieved the final operation is removal of material from regions of high and low relative concentration.

Most of the classical methods of purifying substances present in the same phase have involved phase changes or diffusional separation with semipermeable membranes in which a very high degree of enrichment was attainable in a single operation. It was upon a high enrichment factor that attention was concentrated, for when the factor was small, purification became an intolerably tedious and laborious process. Simple distillation many times repeated, or fractional crystallization carried out in long rows of dishes, are the examples perhaps most familiar.

Much of the apparatus of a normal laboratory, e.g. for filtration, distillation, centrifugation, is concerned with the separation of phases, within one of which the desired substance has become relatively enriched. But for a long time past and increasingly in the last few years, apparatus has been devised in which equilibration between phases can be many times repeated in a countercurrent manner, so that without performing separate operations, a relatively small enrichment factor gives rise to good separation. Absorption and extraction towers, distillation columns and countercurrent liquid-liquid extraction columns are common examples of such apparatus. This countercurrent principle is equally applicable to many diffusional separations, and has been widely used in isotope separations by diffusional and thermodiffusional methods. The first demonstration of the latter method by Clusius & Dickel (1938) had a brilliant and elegant simplicity.

The chromatogram is another simple and elegant, and much older, example of the application of the countercurrent principle to purification. But, in spite of its venerable history, it is still too modern for many laboratory and particularly industrial workers, who remain convinced of the virtues of stirring up the adsorbent with the liquid to be treated.

The examples of countercurrent apparatus given above cover most of the possible varieties of equilibrium between gaseous, liquid and solid phases. In one most important field however, that of fractional crystallization, no apparatus giving countercurrent enrichment has been produced, at least so far as the writer is aware. The problem does not theoretically seem to be insoluble. It seems probable that it is not necessary that crystals should be completely dissolved and reprecipitated; if they are finely enough divided washing is all that is required. If this be true one of the major problems of initiating crystallization in a reproducible way could be avoided.

Countercurrent apparatus if efficient in the widest sense involves the use of many stages of equilibration, or of their equivalent in continuous columns. The time taken to pass such 'theoretical plates' is therefore important, otherwise highly efficient apparatus will be too slow to be

used. Rapid equilibration can only be obtained by reducing the distance over which the molecules must diffuse to a minimum, and increasing the interface between the phases to a maximum. In fluid phases turbulence can reduce the diffusion distance. In solid or stationary fluid phases thin films or small particles must be employed. In the chromatogram the use of fine particles offers no obstacles, and fine particles, it should be noted, reduce the diffusion distance in the mobile phase as well as in the stationary phase. If the particles are large, clear-cut zones cannot be obtained, but this point is not appreciated to a surprising extent. Manufacturers and users of ion exchange resins often recommend and use particles of excessive size. There are, of course, adsorbents in which equilibration is slow even when they are finely divided; active carbon is a good example, and if the movement of a substance from one phase to another involves an activation energy this will always limit the rate of equilibration. If, for instance, tautomerization occurs, this will probably determine how fast a column (of any kind) can be operated. It is perhaps worth pointing out here that in the case of 'Frontal Analysis' due to Tiselius (1940), slow attainment of equilibrium is relatively less important than in other ways of using a chromatogram. The front may still be readily detected, though the estimation of quantity may become more difficult. In contrast, a similar slowness in attaining equilibrium may prevent displacement development being used at all.

FACTORS GOVERNING ENRICHMENT

Let us now turn to the enrichment factor that can be obtained by passing from one phase to another in equilibrium with it. If we restrict our discussion to ideal solutions, i.e. those obeying Raoult's law,

$$\mu_A^S = \mu_A^{S_0} + RT \ln N_A^S,$$

where μ_A^S is the chemical potential of the substance A, $\mu_A^{S_0}$ is the chemical potential in some defined standard state, and N_A^S is the mole fraction of A in the phase S.

If two phases S and M are in equilibrium the chemical potential of all components is, of course, the same in each. Thus

$$\mu_A^M - \mu_A^S = 0 = \mu_A^{M_0} - \mu_A^{S_0} + RT \ln N_A^M - RT \ln N_A^S,$$

or if

$$\mu_A^{S_0} - \mu_A^{M_0} = \Delta\mu_A,$$

$$\Delta\mu_A = RT \ln \left(\frac{N_A^M}{N_A^S} \right).$$

N_A^M / N_A^S is the partition coefficient (expressed in terms of mole fractions) $= \alpha$,

$$\ln \alpha = \frac{\Delta\mu_A}{RT},$$

and $\Delta\mu_A$ is equal to the free energy required to transport one mole of A from phase S to phase M.

Now to a first approximation $\Delta\mu_A$ may be regarded as made up of

$$d\Delta\mu_{CH_2}+e\Delta\mu_{COO^-}+f\Delta\mu_{NH_3^+}+g\Delta\mu_{OH}+\ldots, \text{ etc.},$$

the sum of the potential differences of the various groups of which the molecule A is composed. That is to say, to a first approximation the free energy required to transport a given group, e.g. CH_2, from one solvent to another is independent of the rest of the molecule. Thus all isomers containing the same groups (note that the degree of ionization, etc., must not be changed) would be expected to have the same partition coefficient.

Now, if we consider the partition coefficients α_A and α_B of two substances A and B which differ in that B contains, in addition to those contained in A, a group X, we have,

$$\ln\alpha_A=\frac{\Delta\mu_A}{RT},\quad \ln\alpha_B=\frac{\Delta\mu_B}{RT}+\frac{\Delta\mu_X}{RT},\quad \ln\left(\frac{\alpha_B}{\alpha_A}\right)=\frac{\Delta\mu_X}{RT}.$$

Thus the addition of a group X changes the partition coefficient by a given factor depending on the nature of the group, and on the pair of phases employed, *but not on the rest of the molecule.*

This is a prediction contrary to usual expectation. It is usually felt that the formation of a derivative of greatly increased molecular weight will 'swamp' any differences that exist and will render separation more difficult. This, however, is not to be expected if such a derivative be chosen that the same pair of phases can be employed while still maintaining convenient values for the partition coefficients.

Let us apply this rule to amino-acids and peptides. On the formation of a dipeptide molecule from two amino-acid molecules, or a tripeptide from an amino-acid and a dipeptide, one —CONH— group is created and one COO^- and one NH_3^+ are destroyed. Let the amino-acids be $A^{NH_3^+}_{COO^-}$ and $B^{NH_3^+}_{COO^-}$ and the peptide $NH_3^+.A.CO.NH.B.COO^-$, and let the partition coefficients be α_A, α_B and α_{AB} respectively,

$$RT\ln\alpha_A=\Delta\mu_{NH_3^+}+\Delta\mu_A+\Delta\mu_{COO^-},$$

$$RT\ln\alpha_B=\Delta\mu_{NH_3^+}+\Delta\mu_B+\Delta\mu_{COO^-},$$

$$RT\ln\alpha_{AB}=\Delta\mu_{NH_3^+}+\Delta\mu_A+\Delta\mu_{CONH}+\Delta\mu_B+\Delta\mu_{COO^-},$$

$$RT\ln\left(\frac{\alpha_A\alpha_B}{\alpha_{AB}}\right)=\Delta\mu_{NH_3^+}+\Delta\mu_{COO^-}-\Delta\mu_{CONH},$$

i.e. the product of the partition coefficients of the constituent amino-acids divided by the partition coefficient of the dipeptide is a constant for any given phase pair.

The predictions of this rule hold nearly as well as that for isomers. Thus a peptide *AB* should have the same value as peptide *BA*, and, in fact, they seldom differ by as much as 30%, while the range of partition coefficients covered by the rule may be very large indeed (factors of thousands).

The ratio of solubilities of amino-acids in water and in various organic solvents has been treated similarly by Cohn & Edsall (1943). They extend the treatment to non-ideal solutions and give much experimental data.

It follows from the argument above that the chemical potential for a large molecule is correspondingly large. Hence the difference in potential in moving from one solvent to another, or to a crystal of the substance, will be very sensitive to changes in the solvent. This does not mean that it is easy to separate large molecules differing slightly by salting-out techniques where small changes in the solvent suffice to cause precipitation, but that where such techniques do succeed in purification, it is because there are large differences in the molecules.

A large enrichment ratio of one substance relative to another, on moving from one phase to another, implies a large difference in the changes of chemical potential of the two substances. If the molecules are closely similar such a difference can occur only if the two phases themselves are dissimilar in composition. In general, therefore, phases should be chosen as far as possible from critical solution composition; this approach is in most cases limited by insolubility of the substances under investigation or inconveniently high or low values of the partition coefficient.

It is possible in many cases to make a plausible guess as to the nature of the forces which are important in determining the partition coefficient. If we consider, say, a CH_2 group passing from water to *cyclo*hexane, the CH_2 group will have van der Waals forces between it and the water and between it and the *cyclo*hexane, probably not greatly different in magnitude, and it will also, while in the water, separate water molecules and in the *cyclo*hexane separate *cyclo*hexane molecules. Whereas the *cyclo*hexane molecules are attracted together only by van der Waals forces, the water molecules are attracted also by the relatively powerful hydrogen bonds. The hydrogen bonding of the water molecules to each other will thus be responsible for the high partition in favour of the *cyclo*hexane.

The van der Waals forces can be to some extent specific. Thus benzene will favour aromatic more than aliphatic substances, and in *cyclo*hexane this is reversed. Now the similarity of the melting- and boiling-points of these solvents show that the van der Waals forces are very similar in magnitude in them, but because of similarity of shape of some part of

it, an aromatic molecule may be expected to fit closer to a benzene than a *cyclo*hexane molecule and hence the van der Waals forces will be greater. The same argument applies, *mutatis mutandis*, to aliphatic solutes. Benzyl alcohol, compared to butanol, shows a similar preference for aromatic substances. The great affinity of active carbon for aromatic rings is noteworthy, the C—C spacings in graphite being very similar to those in benzene.

This specificity is a kind of pale reflexion of that so common in crystallization. When crystallization is a useful method of purification, it is because a foreign molecule cannot fit into or on to the crystal so closely, and hence the van der Waals or other forces holding the foreign molecules are not so great as those holding the molecules of the crystal proper. (As a kind of converse of this, crystal specificity might be used chromatographically; a column of some finely divided more or less soluble crystal, which could form mixed crystals with one of the substances to be isolated, being employed.)

For steric reasons the solvent molecules will in many cases be unable to approach closely, and this will reduce the energy of association of the solute with the solvent below that expected from consideration of the sum of the various chemical groups of the solute molecule. Large compact molecules, e.g. native proteins, are extreme examples. Such is, no doubt, the explanation of many of the deviations from the rules discussed above; various isomeric sugars or peptides can be separated on partition chromatograms which would be expected from the rules to be inseparable. Steric factors will be even more important in adsorption, where only one side of the molecule can be expected to be in contact with the adsorbent. It is therefore to be expected that adsorption chromatography will, in general, be more successful in the separation of isomers than partition chromatography.

Hydrogen bonds (Pauling, 1945) between the solute and the solvents are of great importance in determining the partition coefficient. They are far stronger than van der Waals forces (unless many atoms are concerned in the latter). Phenol and collidine, when saturated with water, are excellent solvents for substances capable of forming hydrogen bonds.

They are of opposite character in that phenol is a proton donor, while collidine is a proton acceptor. Water, of course, is both a proton donor and acceptor. Thus we find that the addition of an amino group has little influence on the partition between phenol and water, while it greatly changes the partition between collidine and water, in favour of the water. The amino group is a proton acceptor. Collidine, on the other hand, can accept a proton from an hydroxyl group, and thus the addition of an hydroxyl group makes little difference to the partition

between collidine and water, whereas an hydroxyl group displaces molecules from phenol to water. The carboxyl group contains both the proton-donating hydroxyl and the proton-accepting carbonyl group, and behaves, if the effects of ionization be allowed for, as would be expected, the hydroxyl character predominating.

In partition chromatograms using phenol as solvent, proline runs faster than valine. Using butanol, valine is faster than proline. Here the imino group of proline is a stronger proton acceptor than the amino group of valine, and phenol is a stronger proton donator than butanol. The more acidic character of phenol than of butanol and the more basic character of the nitrogen of proline than that of valine are further examples of the proton-donating or accepting character of these molecules. A similar phenomenon is shown by acetylproline and acetylvaline in chloroform and in *cyclo*hexane. Here the chloroform is the proton donor (cf. its formation of a maximum boiling azeotrope with the proton-accepting acetone) and acetylproline and acetylvaline run at the same speed. In *cyclo*hexane (or ethyl acetate) the acetylvaline runs very much faster.

Ionic bonds are stronger than hydrogen bonds. Where substances are weak acids or bases the degree of ionization is under control by changing the pH; since the partition in most solvent pairs for ionized molecules is very heavily in favour of the more polar phase, the overall partition coefficient is under control simply by changing the pH. When two substances of different pK are concerned the enrichment ratio is similarly a function of the pH. Partition chromatograms utilizing buffers as the stationary phase are now well established, and will be considered further below.

It is premature at the present time to attempt to give quantitative data for free-energy changes on moving various groups from one phase to another for the solvents commonly used in partition chromatography. Cohn & Edsall (1943, p. 212) have given values for the change between water and various liquids, e.g. formamide, methanol, ethanol, butanol and acetone. The values for the CH_2 group for, say, the collidine/water equilibrium phases (which are very temperature-sensitive, being near the critical solution temperature) will be smaller than those in the table given by Cohn & Edsall because of the high solubility of water in the collidine. The same is true to a less extent of the phenol/water equilibrium phases. Rough values for ΔF can readily be deduced from the table of R_F values given by Consden, Gordon & Martin (1944) if the assumption of a given ratio of solvent and water on the paper is made.

When silica gel, cellulose, starch or a synthetic resin is used to support the stationary phase of a partition chromatogram, it will only

in special cases be possible to eliminate adsorption on the molecules of the supporting solid. Thus in the first work with acetylamino-acids on silica gel (Martin & Synge, 1941), it was found that an alcohol of some kind had to be present to the extent of a few per cent in the chloroform before even approximate agreement could be obtained between the expected and the experimental R values. In the case of acetylphenylalanine and acetylleucine, it was very difficult to prepare sufficiently non-adsorptive precipitated silica.

In the case of starch and cellulose, Moore & Stein (1948) have demonstrated that adsorption of various amino-acids is appreciable and does, in fact, at least largely account for the deviations of the R values from those expected from the partition coefficients between water and the mobile solvent. Since hydrogen bonds play so large a part in determining the partition, it is to be expected that hydrogen bonds between the hydroxyl groups of the carbohydrate of the supporting solids and the solute will also contribute. The question as to whether or not adsorption occurs in a given case is, of course, of academic interest; it is not necessarily of practical interest. If the adsorption isotherm is linear no extra spreading of the zones will occur, and the adsorption may lead either to an increase or to a decrease of the separation of the zones.

It is possible to use kieselguhr instead of the supporting solids mentioned above. For very many substances kieselguhr is practically non-adsorbent, the skeletons of the diatoms consisting of relatively dense silica, not much more adsorbent than powdered quartz. The stationary phase is held between the spines and in the holes, essentially in droplet form, not as in silica gel in a network of submicroscopic crystals. It is possible that in most cases in which kieselguhr is the supporting solid for partition chromatograms they will behave pretty accurately as pure partition chromatograms. Further, and by far more important, their use should render possible partition chromatography of large molecules, proteins, polysaccharides and synthetic polymers, which could not be expected to be able to enter silica gel, or starch. It would not of course be surprising if, because of the large size of the molecule, adsorption were appreciable.*

* Kieselguhr columns cannot be packed as a slurry in the mobile phase as silica gel can, by simply pouring it into the chromatogram tube. The kieselguhr after mixing with half its weight of stationary phase (with a spatula in a beaker), can be packed with a ramrod into the tube. This method gives indifferent columns. A much better method is to slurry the mixed kieselguhr and stationary phase with enough mobile phase to give a creamy consistency. The slurry is poured into the tube and thoroughly homogenized by a few rapid strokes of a perforated disk, which is mounted by its centre on a long wire handle. The disk should fit the tube closely. The disk is brought to within about an inch of the bottom of the tube and then moved slowly downwards. This causes the kieselguhr to pack beneath it. Rapid strokes followed by a slow packing stroke are repeated until

Tiselius (1940) was the first to show clearly that chromatograms may be run in three distinct ways, viz. frontal analysis, elution development and displacement development. These methods of running are as applicable to partition as to adsorption chromatograms.

In elution development the permissible loading of the column is usually rather low, as the partition coefficient does not often remain constant over a wide range of concentration, and any change in the partition coefficient leads to a corresponding increase in the widths of the zones.

Displacement development can only occur when changes in partition coefficient occur in the presence of the solute of the following zone. In columns loaded with buffer or strong acids or bases a gradient of pH leads to displacement of weak bases or acids.

Since constancy of partition coefficient is not desirable in displacement columns high concentrations can be used, and the amount of material that can be handled on a column is very large. Adsorption is usually quite unimportant, even where in the corresponding elution column it is troublesome.

Since in displacement columns one zone follows immediately upon the heels of another, good separation demands that the column runs with the utmost uniformity. Complete separation on the column is valueless if the zones are so distorted that they cannot be cleanly separated. In elution development a zone of zero concentration can cover a certain amount of distortion. But Claesson (1947) and Hagdahl (1948) of the Tiselius school have worked out a technique whereby excellent separation may be achieved. The column is divided into several sections connected in series by short fine-bore tubes. Any irregularity in one section (within limits) can be corrected in the section below.

the whole column is packed. If the kieselguhr and stationary phase mixture is heavier than the mobile phase, a funnel with a wide tube attached to the top of the chromatogram tube will save constant refillings. If the mobile phase is the heavier a cork in the top of the chromatogram tube with a hole in it just large enough to provide easy passage for the handle of the disk is a useful aid. The columns when packed are robust and free from gas. Excess liquid may be poured off without fear of disturbance, and their regularity is at least as good as a precipitated silica column, and their efficiency in terms of a height of equivalent theoretical plate is probably higher. There is more resistance to flow of solvent through the column and a few pounds pressure is usually desirable. Whether vacuum or pressure be employed it must be emphasized that the solvent must be kept degassed if trouble with the separation of gas in the column is to be avoided. With thoroughly degassed solvents, gas always disappears from the column even if originally present.

The size of the holes in the perforated disk should be governed by the interfacial tension between the phases. Where this is high $\frac{1}{16}$ in. holes are satisfactory, when low smaller holes should be used, or packing becomes extremely slow. The interfacial tension probably governs also the proportion of stationary phase to kieselguhr that is satisfactory. When the interfacial tension is high more than one of stationary phase to two of kieselguhr may be used.

Possibly kieselguhr can also be used for reversed phase columns if it can be made 'unwettable' by treatment with a suitable silicone, or in some other way.

This technique is available only for frontal analysis and displacement development. If applied to an elution development column it would result merely in substituting a uniform column with poor separation for a non-uniform column with good separation without altering the degree of separation actually obtained. Displacement of material that has lagged behind is the essential part of the technique. The extent of the correction that can be made in each section decreases as the substances themselves become more difficult to separate.

THEORY OF PARTITION DISPLACEMENT CHROMATOGRAMS

The theory of displacement development has been developed only for adsorption columns. A rather different treatment seems desirable for partition columns, and though they could be considered as essentially continuous, the theoretical plate method is easier to understand (cf. Martin & Synge, 1941; Mayer & Tompkins, 1947).

We shall consider below displacement chromatograms loaded with NaOH or buffer. We shall assume that weak acids A, B, etc., are on the column which is being developed with another weak acid D, and that development has proceeded far enough for a steady state to have been reached. It is assumed that no ionized acid is present in the mobile phase.

Nomenclature

A^M = concentration of acid in mobile phase.

HA = concentration of unionized acid in stationary phase.

A^- = concentration of ionized acid in stationary phase.

$A = A^- + HA$ = concentration of total acid in stationary phase.

$\alpha = HA/A^M$ = partition coefficient of unionized acid. Similarly for various forms of acids B and D, and buffer acids P_1, P_2 assumed insoluble in the mobile phase.

H^+ = concentration of hydrogen ions in stationary phase.

$K_A = \mathrm{H}^+A^-/HA$ = dissociation constant in acid.

M = cross-sectional area of mobile phase in column.

S = cross-sectional area of stationary phase in column.

T = cross-sectional area of total area of column.

R = ratio of rate of movement of zones divided by rate of movement of developing liquid in tube above column.

V_M = volume of mobile phase which passes a given zone in unit time.

V_S = volume of stationary phase which is passed by given zone in unit time $V_M/V_S = (T - RM)/RS$.

x = number of equivalent theoretical plates from top of column.

A_x = concentration of acid A in stationary phase leaving plate x.

$A^M_{(x-1)}$ = concentration of acid A in mobile phase leaving plate $(x-1)$.

Considering first the equilibrium between acid in mobile and stationary phases we may write

$$Na^+ + H^+ = OH^- + A^- + B^- + P_1^- + P_2 + \ldots, \text{ etc.} \qquad (1)$$

If the concentration of the buffer is within practical limits and the acids not exceptionally strong or weak, H^+ and OH^- may be neglected; then

$$Na^+ = K_A \frac{HA}{H^+} + K_B \frac{HB}{H^+} + K_P \frac{HP}{H^+}, \ldots, \text{etc.},$$

or

$$Na^+ = \alpha K_A \frac{A^M}{H^+} + \beta K_B \frac{B^M}{H^+} + \frac{K_p P_1}{H^+ + K_{P_1}} + \frac{K_p P_2}{H^+ + K_{p_2}}. \qquad (2)$$

Now consider a cross-section of the column, moving down the column at the same rate as the zones move. Since the column is in a steady state the amount of any of the substances forming the zone (i.e. any of the acids being separated, not those of the buffer), which is left behind in the stationary phase by the cross-section as it moves, is equal to the amount of that substance which passes through the cross-section in the opposite direction in the mobile phase. If the cross-section is between theoretical plates $(x-1)$ and (x) we have the material balance equation

$$V_S A_x = V_M A^M_{(x-1)}, \qquad (3)$$

and similar equations for each species of acid involved.

Now in a region of constant composition such as the middle of zone A

$$A^M_{(x-1)} = A^M_x = A^M_{x+1} \ldots, \text{ etc.}$$

Hence

$$\left.\begin{aligned} &V_S A = V_M A^M, \\ &\frac{V_M}{V_S} = \frac{A}{A^M} = \frac{\alpha (HA + A^-)}{HA} = \alpha\left(1 + \frac{K_A}{H^+}\right), \\ &H^+ = \frac{K_A}{V_M/\alpha V_S - 1} = \frac{\alpha K_A SR}{T - R(M + \alpha S)}, \end{aligned}\right\} \qquad (4)$$

i.e. the pH is determined by the R value and the partition coefficient and dissociation constant of the acid concerned, and is independent of the buffer. (R is, of course, a function of the buffer.)

From equations (2) and (4), remembering that B^M is zero in band A, A^M may be calculated.

Substituting D^M, the developing acid for A^M, the strength of developing acid to give a given R may be found, knowing the concentrations, dissociation constants of the buffer acids, the dissociation constant and partition coefficient δ of the displacing acid and the relative volumes of the phases in the column. From R and the dissociation and partition coefficients of displaced acids the pH and concentration in any zone of constant composition below may be found.

In the special case of a NaOH column

$$R = \frac{T}{S(\mathrm{Na}^+/D^M + \delta) + M}. \tag{5}$$

CONDITIONS IN BOUNDARIES BETWEEN ZONES

Let us first define a layer across the column equivalent to a 'theoretical plate' (Martin & Synge, 1941). In such a layer the mobile phase leaving the bottom of the layer would be in equilibrium with the stationary phase at the top of it. A_x is in equilibrium with A_x^M. Similarly B_x is in equilibrium with B_x^M. (Strictly speaking A and B will have different diffusion constants, and the size of the equivalent theoretical plates will be different. This difference will be neglected and will not lead to any serious error.)

These theoretical plates may be considered as stationary or as moving at any convenient speed in the column. We will let them keep pace with the zones, since then no change in composition will occur within any plate.

Now by definition the liquids issuing from either side of the theoretical plate are in equilibrium. The ratio of A and B in the two phases can be found thus in plate x:

$$A_x^M = \frac{HA_x}{\alpha} = \frac{\mathrm{H}_x^+ A_x^-}{\alpha K_A} = \frac{\mathrm{H}_x^+}{\alpha K_A}(A_x^- - HA_x) = \frac{\mathrm{H}_x^+}{\alpha K_A}(A_x - \alpha A_x^M),$$

$$A_x^M = \frac{A_x \mathrm{H}_x^+}{\alpha K_A + \alpha \mathrm{H}^+}, \quad \text{similarly} \quad B_x^M = \frac{B_x \mathrm{H}_x^+}{\beta K_B + \beta \mathrm{H}^+}$$

$$\frac{A_x^M}{B_x^M} = \frac{A_x}{B_x}\frac{\beta}{\alpha}\left(\frac{K_B + \mathrm{H}_x^+}{K_A + \mathrm{H}_x^+}\right), \tag{6}$$

and H^+ may be found from equation (2) if both A_x^M and B_x^M be known. Now equation (3) in terms of ratios of A^M and B^M and A and B is

$$\frac{A_{(x-1)}^M}{A_x} = \frac{V_S}{V_M} = \frac{B_{(x-1)}^M}{B_x}, \quad \frac{A_{(x-1)}^M}{B_{(x-1)}^M} = \frac{A_x}{B_x}. \tag{7}$$

Starting from any given concentrations of A_x^M and B_x^M we can in theory work from plate to plate and determine the concentration at any point in the column. Exact calculation is tedious, since H_x must be determined at each step. However, a good approximation is readily obtainable. Let us express equations (6) and (7) in logarithmic form. Let us plot them as in Fig. 1, where the vertical distance between the two lines is $\log\frac{\beta}{\alpha}\left(\frac{K_B + \mathrm{H}^+}{K_A + \mathrm{H}^+}\right)$. Now where $\log A/B < -2$, H^+ is that reigning in zone B. Where $\log A/B > 2$, H^+ is that reigning in zone A, and in those regions the curve will be parallel to the lower line expressing the material balance equation. The number of plates required

to effect a given change in ratio of A^M/B^M is represented by the number of steps that can be drawn between the lines, each point on the upper curve representing conditions in one plate.

It is obvious that replacing the centre sigmoid part of the curve by a straight line will give a good approximation so far as the number of steps is concerned, particularly in that the equations are symmetrical in A and B and $\frac{\beta}{\alpha}\left(\frac{K_B+\mathrm{H}^+}{K_A+\mathrm{H}^+}\right)$ will never differ much from unity in the cases in which we are interested.

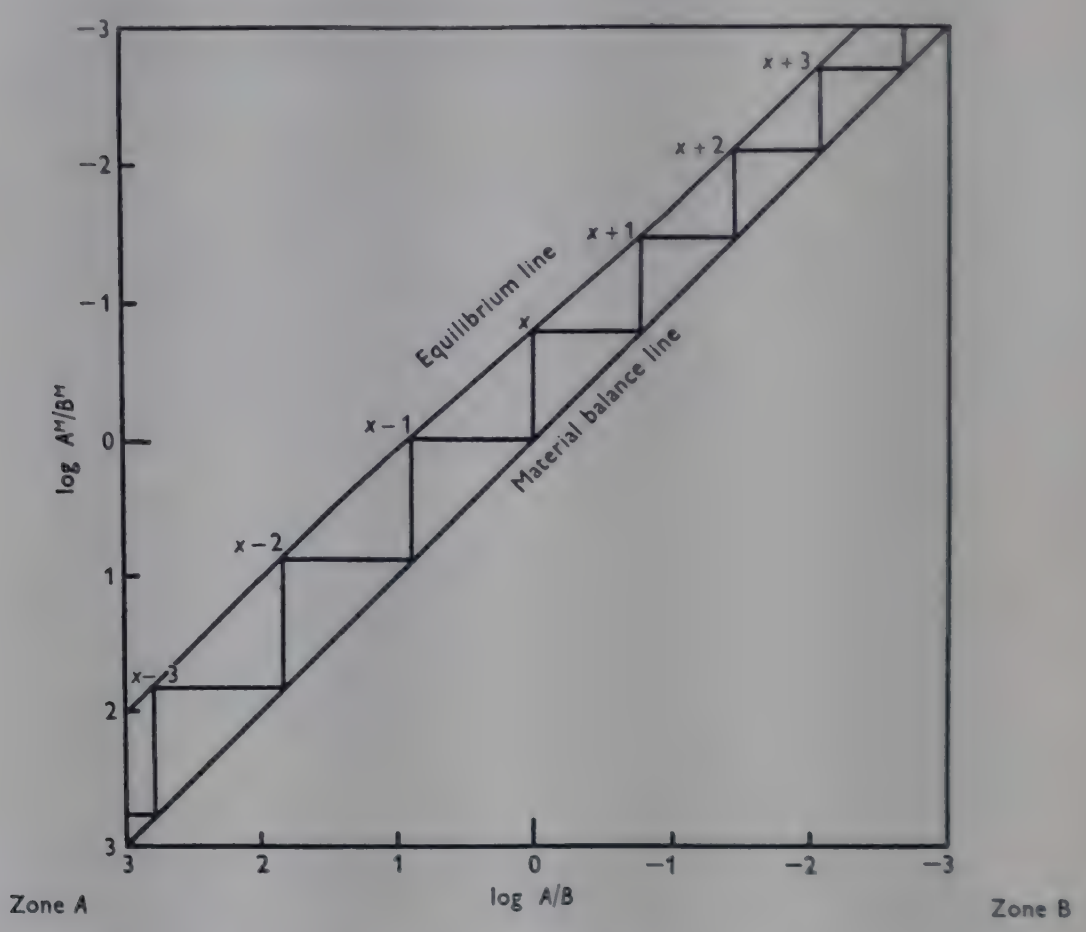

Fig. 1. Diagram showing relationship between number of theoretical plates and concentration in mobile and stationary phases.

From the argument above it is obvious that a good approximation will be given by

$$\log\left(\frac{A^M_{x+n}}{B^M_{x+n}}\right)-\log\left(\frac{A^M_{x-n}}{B^M_{x-n}}\right)=n\log\frac{\beta}{\alpha}\left(\frac{K_B+\mathrm{H}^+_A}{K_A+\mathrm{H}^+_A}\right)+n\log\frac{\beta}{\alpha}\left(\frac{K_B+\mathrm{H}^+_B}{K_A+\mathrm{H}^+_B}\right)$$

if x is the plate where $A=B$. H^+_A and H^+_B are the hydrogen-ion concentrations in zones A and B, and $2n$ is the number of plates required.

It should be noticed that the conditions in the column, and the mathematical treatment, are essentially similar to those in a distillation column run at total reflux.

Some examples of simple cases may be helpful here.

Example 1. Consider a NaOH column. Let

$K_A = K_B = 10^{-5}$, $\alpha = 0{\cdot}2$, $\beta = 0{\cdot}1$, $Na^+ = 0{\cdot}1$ normal $V_M/V_s = 1$:

$$H_A^+ = \frac{K_A}{V_M/\alpha V_s - 1} = \frac{10^{-5}}{5-1} = 2{\cdot}5 \times 10^{-6}, \quad pH_A = 5{\cdot}60,$$

$$H_B^+ = \frac{K_B}{V_M/\alpha V_s - 1} = \frac{10^{-5}}{10-1} = 1{\cdot}11 \times 10^{-6}, \quad pH_B = 5{\cdot}95.$$

Difference in pH in two zones = 0·35 unit. Concentration in zone A

$$A^M = \frac{Na^+ H_A^+}{\alpha K_A} = 0{\cdot}125 \text{ normal},$$

in zone B $$B^M = 0{\cdot}111 \text{ normal}.$$

In this case, since $K_A = K_B$, the enrichment ratio $\frac{\beta}{\alpha}\left(\frac{K_B + H^+}{K_A + H^+}\right)$ is $\frac{\beta}{\alpha}$ and is independent of pH.

The number of plates required to change the concentration ratio A^M/B^M from 0·001 to 1000 is

$$2n = \frac{\log\left(\frac{A^M_{x+n}}{B^M_{n+x}}\right) - \log\left(\frac{A^M_{x-n}}{B^M_{x-n}}\right)}{\log\frac{\beta}{\alpha}} = \frac{3+3}{0{\cdot}301} = 20.$$

Had α/β been 1·1 instead of 2·0, then $6/\log 1{\cdot}1 = 145$ plates would have been needed.

Example 2. NaOH column. Let

$K_B = 10^{-5}$, $K_A = 2 \times 10^{-5}$, $\alpha = \beta = 1$, $Na^+ = 1$ normal $V_M/V_s = 1$,

$H_A^+ = 2{\cdot}22 \times 10^{-6}$, $H_B^+ = 1{\cdot}11 \times 10^{-6}$, $pH_A = 5{\cdot}65$, $pH_B = 5{\cdot}95$,

$$\log\frac{\beta}{\alpha}\left(\frac{K_B + H^+}{K_B + H^+}\right) = -0{\cdot}279 \text{ in zone } B$$

$$= -0{\cdot}260 \text{ in zone } A,$$

$$A^M \text{ in zone } A = 0{\cdot}111N = B^M \text{ in zone } B.$$

Number of plates required to change A^M/B^M from 0·001 to 1000

$$= \frac{2 \times 6}{0{\cdot}279 + 0{\cdot}260} = 22.$$

Thus we see that for practically complete separation where the enrichment ratio is 2 only twenty plates are required. When it is 1·1 about 150 plates are required.

Now it is difficult to separate substances differing in enrichment ratio by 1·1 by elution development, and though no figures are available for the height of the equivalent theoretical plate in buffered columns there seems no reason to expect it to be greatly different from the water-laden silica columns (Martin & Synge, 1941). In that case complete separation

is to be expected in a few millimetres.* (However, in ion-exchange resin columns, the height of the theoretical plate appears to be much greater (Mayer & Tompkins, 1947).)

The distance which a displacement column must be run before a steady state is reached cannot be simply calculated. As the substances to be separated are first run into the column frontal analysis occurs. Though qualitatively it is easy to see what happens, the equations for three substances on a buffered column are troublesome to solve. While the transition to displacement development occurs as the developing acid flows in, the conditions become far more complicated, since some diffuse as well as sharp fronts will occur.

A rough approximation may be made as follows. Assume that two of the substances A and B are much more difficult to separate than the others. When displacement development is complete let them occupy lengths a and b of the column and let the pH in their respective zones be pH_A and pH_B. Equation (4) may be written as

$$R_A = \frac{T}{M + \alpha S\,(1 + K_A/\mathrm{H}^+)}, \quad R_B = \frac{T}{M + \beta S\,(1 + K_B/\mathrm{H}^+)}.$$

If a value for H^+ be assumed equal to $\frac{1}{2}(\mathrm{H}_A^+ + \mathrm{H}_B^+)$ (which should be a fair average), then the distance of the front of A from the top of the column should be approximately $\dfrac{a+b}{R_B - R_A}$ plus the width of any zones slower than A.

The question may now be raised as to optimum pH to aim for to get sharpest separation and the relative advantages of using NaOH or buffer columns.

When $\beta/\alpha < 1$ and $K_B/K_A < 1$, a high pH is desirable.

When $\beta/\alpha < K_A/K_B < 1$, a low pH is desirable.

When $K_A/K_B < \beta/\alpha < 1$, a high pH is desirable.

In all cases better separations can be expected with NaOH than with buffer, since larger pH changes occur down the column. (When the relative proportion of buffer to A and B, etc., becomes large, elution development rather than displacement development will occur. We shall not discuss the relative efficiencies of separation by these two methods.)

It should be noted that buffer offers little advantage in choosing the pH at which the column should run. A suitable ratio of concentration of developing acid and NaOH can select any desired pH for a given zone.

A buffered column may offer advantages in the detection of the passage of fronts, since a rise of concentration in the mobile phase can usually be expected as each front passes. With NaOH columns, these

* Added in proof. This figure is too optimistic, by at least a factor of ten, for both buffered and unbuffered columns.

changes in concentration may be positive or negative (they depend solely on α, β, etc., not on the dissociation constant).

On the other hand, if pH changes be used to detect the passage of the fronts, NaOH columns will be the more sensitive. A 10% change in partition coefficient or dissociation constant should give rise to a change of about 0·04 pH unit, which should be detectable with suitable apparatus.

Using conductivity changes in the stationary phase a buffer column may be better if there is a big difference between the mobilities of the buffer ions and the ions of the acids to be separated.

OTHER POSSIBLE TYPES OF DISPLACEMENT CHROMATOGRAMS

Displacement development has so far been used only on buffer, NaOH or acid-loaded columns. If, however, systems can be found where one phase contains a limited amount of substance which will bond with the solutes, the condition for displacement development exists. Chelation is an obvious suggestion.

As a kind of converse of this it has been found (Baker, Dobson & Martin, 1948) that paper partition chromatograms of the hydroxamic acids derived from penicillins gave streaky spots, presumably due to formation of chelated double molecules in the mobile phase, resulting in a variable partition coefficient. By using citric or phthalic acids which are slightly soluble in the mobile phase for the buffer, instead of phosphoric or oxalic acids which are insoluble, chelation in the mobile phase occurs between the hydroxamic acids and the citric or phthalic acids. Because the buffer acid is present in the mobile phase in a relatively large excess, the partition coefficients of the hydroxamic acids are displaced in favour of the mobile phase and remain constant over a much wider range of concentration, the spots being rendered compact.

In closing, a rather speculative suggestion will be made for a possible variety of chromatogram using homopolar bonds, in place of ionic or hydrogen bonds or van der Waals forces. The bond suggested is an ester link, and the stationary phase would contain a polyvinyl alcohol if acids were to be separated, or a polyacrylic acid if alcohols were to be separated. The stationary phase must also contain a strong acid as a catalyst. The mobile phase would consist of a hydrocarbon or ether. Though a catalyst were present the equilibrium would still be relatively slowly reached, so that the chromatogram would have to be developed extremely slowly.

Such a chromatogram should be capable of either elution or displacement development. In principle there seems no reason why it should not be a success.

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3. APPLICATION OF PARTITION CHROMATOGRAPHY TO THE STUDY OF PROTEIN STRUCTURE

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It is generally recognized that the Fischer-Hofmeister hypothesis of protein structure was the earliest and greatest advance in the field of protein chemistry. Since that time, no single method has given a greater impetus to the purely chemical study of protein structure than the application by Martin & Synge (1941*b*) of partition chromatography for the separation, detection and estimation of amino-acids and peptides. In its scope and adaptability, it is a method which has changed the whole course of many fields of biochemical research, and must be classed as one of the greatest advances of the last decade.

Since Fischer and Hofmeister first demonstrated that proteins consist of chains of amino-acids bound together through the peptide linkage, much painstaking effort has been applied to the overall analysis of proteins, so that at the present time we know with considerable accuracy the number of the different amino-acid residues contained in a few of the more purified proteins. However, we know almost nothing about the way in which these residues are arranged in the peptide chains of the proteins. A knowledge of this arrangement would seem to be of particular importance, as it is probable that the unique physiological properties and specificities of proteins are conditioned by the particular arrangement of amino-acid side-chains in the molecule. Attempts to determine the structure of the catalytically active centres of enzymes and hormones by inhibition methods have given only incomplete results, and it would seem that the only real approach to this general problem of protein structure is through a study of the structure of the products of partial degradation of proteins. Early attempts to isolate peptides from partially hydrolysed proteins were greatly hampered by the complexity of the hydrolysates and the lack of suitable methods for fractionating mixtures of closely related peptides, and it is in this field that partition chromatography is of such great service. Although we still know very little about the order in which the amino-acids are arranged in any protein, methods have been worked out and a beginning made on the separation of peptides. It is probable, indeed, that the complete amino-acid sequence in a protein will be elucidated in the not too distant future.

APPLICATION OF PARTITION CHROMATOGRAPHY TO THE ANALYSIS OF PROTEINS

The acetamido-acid method

Partition chromatography was originally introduced as a method for separating and estimating the acetyl derivatives of the amino-acids, and especially the monoamino-acids. At that time the only technique for estimating this group was the old ester distillation method which was very laborious and not very accurate, so that the new method fulfilled a very necessary purpose and is still one of the most reliable methods for this group, though others have since been developed.

After experimenting with a very complicated apparatus for counter-current distribution separations of the acetamido-acids, Martin & Synge (1941*a*, *b*) found that the whole process could be carried out very simply on a column of wet silica gel. In this method (Gordon, Martin & Synge, 1943*a*) a protein hydrolysate was treated with an excess of acetic anhydride to convert the amino-acids quantitatively to their acetyl derivatives, which were separated by partition chromatography on silica gel and estimated by titration and/or nitrogen determination. The acids were made visible on the column by incorporating a suitable indicator in the stationary aqueous phase of the column (see also Gordon *et al.* 1944; Liddell & Rydon, 1944).

The method was first applied to the antibiotic substances gramicidin (Gordon *et al.* 1943*b*), tyrocidine (Gordon *et al.* 1943*d*) and 'gramicidin S' (Synge, 1945). In the case of gramicidin* and 'gramicidin S' the results, which are summarized in Table 1, were found to be reproducible, and to match the values for a calculated stoichiometric unit.

Table 1. *Amino-acid composition of gramicidin and 'gramicidin S'*

Amino-acid	N as % total N (found)	Assumed no. of residues	Calc. from assumed no. of residues
	Gramicidin (Gordon *et al.* 1943*b*)		
Leucine	20·2	6	20·0
Tryptophan	40–45	6	40·0
Valine	16·6	5	16·7
Alanine	10·1	3	10·0
Glycine	5·3–6·6	2	6·7
	'Gramicidin S' (Synge, 1945)		
Ornithine	33·0	1	33·3
Proline	16·7	1	16·7
Valine	17·1	1	16·7
Leucine	16·8	1	16·7
Phenylalanine	16·6	1	16·7

With tyrocidine, the values for a number of the amino-acids were not so consistent, and this was tentatively put down to the state of linkage of the tryptophan residues.

* See, however, Gregory & Craig (1948) and Synge (1949).

The Leeds workers have emphasized the importance of carrying out parallel analyses of control mixtures and of using a suitable batch of gel, which must have a minimum of adsorptive properties. These two factors have also been emphasized by Tristram (1946), who has used the method extensively for analyses of proteins, and has also emphasized the importance of carrying out a considerable number of replicate analyses since unaccountable factors may give rise to fortuitous results in a single estimation. Tristram has found the method to give accurate results for the analyses of phenylalanine, the leucine isomers, valine, proline and alanine. The proportions of the leucine isomers can be subsequently determined by infra-red spectrometry (Darmon, Sutherland & Tristram, 1948). Tristram's results for the analyses of insulin, edestin and β-lactoglobulin are summarized in Table 2.

Table 2. *The amino-acid composition of insulin, edestin and β-lactoglobulin* (Tristram, 1946)

(Values are N as % total N)

	Insulin	Edestin	β-Lactoglobulin
Phenylalanine	4·4	2·6	2·3
Leucine	7·8	5·0	17·1
Isoleucine	3·2	2·1	
Valine	6·0	3·1	4·45
Proline	2·0	2·6	4·3
Alanine	4·4	3·65	6·1

Paper chromatography

Probably the most useful application of partition chromatography is the method of paper chromatography (Consden, Gordon & Martin, 1944). Here the amino-acids themselves are fractionated on strips of filter paper, the aqueous phase being held by the cellulose, while an organic solvent is allowed to flow down the paper. The development can be carried out first in one direction and then at right angles to that direction using two different solvents, which increases the resolving power of the method. The positions of the amino-acids on the paper can be revealed by spraying with a solution of ninhydrin or by the fluorescence produced by amino-acids themselves in ultra-violet light (Phillips, 1948). On such a two-dimensional chromatogram the naturally occurring amino-acids form a characteristic pattern, and it is easy to tell by inspection if any particular amino-acid is absent or present in unusually large amounts. When only a few amino-acids are present, they can be identified by measuring their R_F values, but it is better to run parallel chromatograms with known amino-acids, since R_F values are not completely reproducible. The most useful solvents for amino-acids are phenol and collidine or lutidine. On a two-dimensional chromatogram with these two solvents it is possible to distinguish all

the naturally occurring amino-acids with the exception of a few of the more fat-soluble ones. These latter can be identified using a mixture of butanol and benzyl alcohol. Other solvent systems have been described (e.g. Edman, 1945), and for certain purposes may be suitable, but the above solvents are probably the most useful for studying unknown mixtures. Collidine has certain disadvantages due to its sensitivity to temperature and its smell, and for most purposes can be replaced by a mixture of butanol and acetic acid (Partridge, 1948). For a complete qualitative analysis of a protein about 0·5 mg. of material and very little labour are required, and this method, which completely replaces most of the older methods of identifying amino-acids, should now be a standard procedure in all laboratories where protein chemistry is studied.

One of the earliest applications of paper chromatography was the demonstration that norleucine probably does not occur in nature. The richest source of this material was considered to be spinal cord, but Consden, Gordon, Martin, Rosenheim & Synge (1945) showed that when a hydrolysate of spinal cord was run on a suitable chromatogram, there was no spot in the expected position.

A special study was made of various sulphur-containing amino-acids in connexion with studies on wool, and it was shown that wool tips contain cysteic acid which presumably arises from cystine by oxidation in the air (Consden, Gordon & Martin, 1946).

Paper chromatography has been used extensively in the study of the polymyxins, a group of polypeptide antibiotics produced by strains of *B. polymyxa* (Jones, 1948*c*). The various polymyxins can be separated on paper chromatograms and distinguished from one another by their R_F values. They also differ qualitatively in their amino-acid make-up. Polymyxin A (aerosporin), which has been studied the most, contained only three amino-acids, which were identified as D-leucine, L-threonine and α, γ-diaminobutyric acid (Jones, 1948*a*; Catch & Jones, 1948). This latter had not been identified previously in any biological material, and its presence was confirmed by isolation. The optical form of the leucine and threonine was demonstrated by a method due to Synge (1949) (cf. Jones, 1948*b*). The developed chromatogram was sprayed with a solution of D-amino-acid oxidase. The D-leucine spot did not appear on subsequent spraying with ninhydrin, as it had been decomposed by the enzyme, whereas the L-threonine was unaffected. By comparing the strengths of colour produced by ninhydrin with that produced with standard amounts of the amino-acids, it was estimated that there was one leucine residue to every three threonine residues. The amino-acid components of the various polymyxins are shown in Table 3. Besides the amino-acids, they also contain an unidentified saturated optically

active fatty acid. Edman (1945) has studied hypertensin by paper chromatography.

Paper chromatography has also been used in the study of insulin structure (Sanger, 1948). Insulin itself can be shown to contain all the usual naturally occurring amino-acids except tryptophan, methionine and hydroxyproline. After splitting the insulin into its separate polypeptide chains by oxidation of the —S—S— bridges, a fraction can be separated which contains no basic amino-acids, threonine or phenylalanine, and it is believed that this represents at least one of the individual chains of the insulin molecule, and demonstrates a rather asymmetric distribution of the amino-acids within the molecule.

Table 3. *The amino-acid components of the polymyxins* (Jones, 1948*c*)

Polymyxin	Leucine	Phenyl-alanine	Threonine	Serine	α, γ-Diamino-butyric acid
A	+	−	+	−	+
B	+	+	+	−	+
C	−	+	+	−	+
D	+	−	+	+	+
E	+	−	+	−	+

Many other applications of paper chromatography to protein chemistry could be quoted (see Consden, 1948). It is certain that many more will be reported in the near future, and that this method will make a great contribution to our knowledge of protein structure.

As originally introduced, paper chromatography was essentially a qualitative method. An approximate assay of the amount of amino-acid present can be gained from the size and depth of colour of the spot produced with ninhydrin, especially if standard solutions of amino-acids are run on parallel chromatograms with the unknown. Indeed, considerable accuracy is claimed for this type of method by Polson, Mosley & Wyckoff (1947), who report a complete analysis of silk fibroin which agrees well with values obtained by other methods. In the study of peptides it is often necessary to determine the number of each amino-acid in a small peptide, and the above rapid method is usually accurate enough for this purpose. More accurate, though rather more laborious, methods have recently been described in which the amino-acid is eluted from the paper and estimated as its copper complex either polarographically (Martin & Mittelmann, 1948) or by the method of Pope and Stevens (Woiwod, 1948).

Other methods of analysis

The ideal basis for an accurate method of amino-acid assay would seem to be a technique in which all the naturally occurring amino-acids can be fractionated completely from one another with a minimum of

chemical manipulation. Paper chromatography would seem to satisfy these requirements, but the amounts of amino-acids required for a good chromatogram are at present too small for an accurate estimation. Elsden & Synge (1944) (cf. Synge, 1944) showed that amino-acids could be separated on a larger scale using columns of starch, and Stein & Moore (1948) (see also Moore & Stein, 1948*a*, *b*) have worked out an almost complete method of fractionation and assay which may prove to be one of the most accurate methods. The eluate from the starch chromatogram is collected in 0·5 ml. samples by means of an automatic fraction taker and the amount of amino-acid in each sample estimated by a quantitative modification of the ninhydrin reaction.*

Another method of analysis depends on the fractionation of the radioactive *p*-iodophenylsulphonyl derivatives on paper chromatograms and estimation of the radioactivity of the spots (Keston, Udenfriend & Levy, 1947). This is an extremely micro technique making possible an analysis on a few μg. of material. It should be capable of considerable accuracy.

STUDIES ON PEPTIDES

As already indicated the most important application of partition chromatography is to the separation of peptides from the products of partial hydrolysis of proteins, and it was with this in mind that the methods were originally developed. An attempt to separate peptides as their acetyl derivatives on columns of silica suggested the presence of a few peptides in gelatin (Gordon *et al.* 1943*c*), but far better results were obtained by the direct fractionation of the peptides on paper chromatograms. A very neat method for the separation and structural analysis of small peptides has been worked out by Consden, Gordon & Martin (1947). The peptide mixture, after preliminary group separations, is fractionated on a number of replicate two-dimensional chromatograms. One chromatogram is used to identify the position of the peptides by spraying with ninhydrin and the peptides are eluted from the others. The structure of each peptide is then determined as follows:

(1) A sample is completely hydrolysed and the amino-acids identified on a suitable chromatogram.

(2) Another sample is deaminated by treatment with nitrosyl chloride and then hydrolysed and put on a chromatogram. The amino-acid which bore the free amino group in the peptide is destroyed by the deamination and does not appear on the second chromatogram.

This technique is especially valuable for dipeptides, as their structures can be completely determined and they move satisfactorily on the chromatograms as compact spots. The structure of higher peptides can also be determined by degrading them to the dipeptide stage, but they

* See also Moore & Stein (1949), Stein & Moore (1949).

tend to form 'tailing' spots on the chromatogram more than the lower peptides.

The first application of this method was to the determination of the order of the amino-acid residues in 'gramicidin S' (Consden, Gordon, Martin & Synge, 1947). The four dipeptides and two tripeptides shown in Table 4 were identified in a partial hydrolysate, and these were sufficient to demonstrate without doubt that the order of the residues in the molecule is: -valyl-ornithyl-leucyl-phenylalanyl-prolyl-. Since there is no free α-amino group (Sanger, 1946) 'gramicidin S' must be a cyclopeptide, and the molecular weight suggests that the above sequence is repeated twice in the molecule. In the more complex polypeptide tyrocidine the following sequences have been worked out (Consden, 1948): -valyl-ornithyl-leucyl- and -aspartyl-glutamyl-tyrosyl-. The complete structure has not yet been reported.

Table 4. *Peptides identified in 'gramicidin S'*
(Consden, Gordon, Martin & Synge, 1947)

Valyl-ornithine	Valyl-ornithyl-leucine
Ornithyl-leucine	Phenylalanyl-prolyl-valine
Leucyl-phenylalanine	Prolyl-valyl-ornithine
Phenylalanyl-proline	

Table 5. *Dipeptides identified from wool*
(Martin, 1946; Consden & Gordon, 1948)

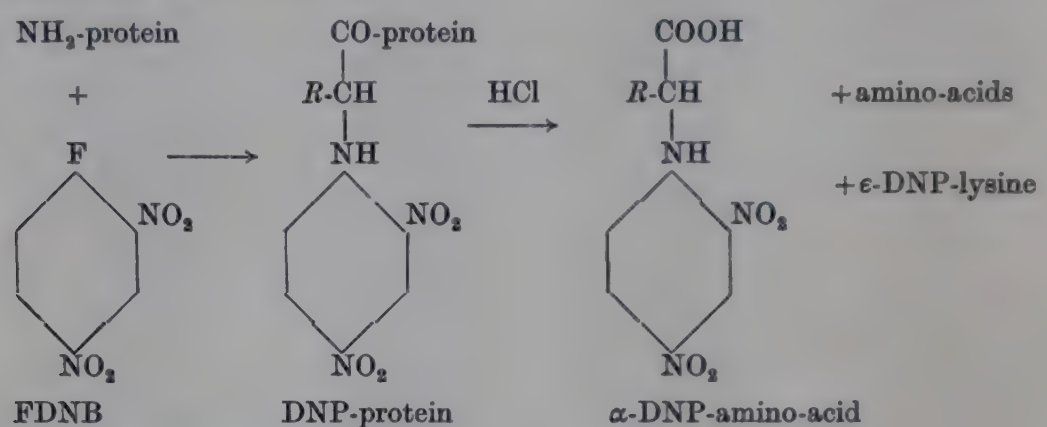
Aspartyl-alanine	Alanyl-aspartic acid	Cysteicyl-glycine
Aspartyl-valine	Valyl-aspartic acid	Cysteicyl-alanine
Aspartyl-leucine	Leucyl-aspartic acid	Glycyl-cysteic acid
Aspartyl-glutamic acid	Glutamyl-aspartic acid	Seryl-cysteic acid
Glutamyl-glycine	Seryl-aspartic acid	Alanyl-cysteic acid
Glutamyl-alanine	Glycyl-glutamic acid	Threonyl-cysteic acid
Glutamyl-glutamic acid	Alanyl-glutamic acid	Cysteicyl-valine
	Leucyl-glutamic acid	Cysteicyl-leucine
	Seryl-glutamic acid	Leucyl-cysteic acid
	Tyrosyl-glutamic acid	Phenylalanyl-cysteic acid

The dipeptides listed in Table 5 have been identified in partial hydrolysates of wool (Martin, 1946; Consden & Gordon, 1948). The acidic peptides were the most studied, and most notable was the very large number of different residues which are linked to glutamic and aspartic acids respectively, indicating a very complex structure for wool. The cysteic acid peptides were obtained from cystine peptides after oxidation of a neutral fraction with bromine. The acidic peptides of cysteic acid, which were formed, could then be separated from unchanged neutral peptides. It could scarcely be expected that the complete structure of such a complex protein as wool could be elucidated by the identification of small peptides, but these investigations have yielded valuable information and have cast considerable doubt on certain speculative theories on the structure of wool.

The possibility of using starch columns for the fractionation of peptides on a rather larger scale has been demonstrated by Synge (1944, 1949), who separated L-valyl-glycine, D-leucyl-glycine, L-alanyl-D-valine and L-alanyl-D-leucine from a partial hydrolysate of gramicidin, and it is to be hoped that this method will be explored further.

FREE AMINO GROUPS OF PROTEINS

Another technique which makes use of partition chromatography is the method for the identification and estimation of the free amino groups of proteins using the reagent 1:2:4-fluorodinitrobenzene (Sanger, 1945; Porter & Sanger, 1948). The principle of the method may be illustrated by the following formulae:



The fluorodinitrobenzene reacts with the free amino groups under mild conditions to form a dinitrophenyl-protein (DNP-protein) which is then hydrolysed to yield DNP-amino-acids. These derivatives are coloured bright yellow and can be fractionated on columns of silica gel, identified by their behaviour on the columns and estimated colorimetrically. It seems that this fractionation is not pure partition chromatography, since the band rates are considerably slowed by adsorption forces (Consden, Gordon, Martin & Synge, 1947). However, it is probable that most of the separations are due to differences in partition coefficient.

The terminal residues of a number of proteins have been determined by this method and the results are summarized in Table 6.

It can be seen that there is considerable variation both in the number of free α-amino groups and in the nature of the amino-acids on which they are located. It is interesting to note, however, that all the terminal residues so far identified belong to the monoamino-acid group. Basic or acidic amino-acids have not yet been detected in the terminal position in any protein. It will be interesting to see if this will prove a general rule for all proteins. The results with a number of haemoglobins (Porter & Sanger, 1948) indicate structural differences between the various species studied and between normal haemoglobin, myoglobin and foetal haemoglobin.

The number of free α-amino groups present in a protein will be equal to the total number of open polypeptide chains, that is to say, chains containing a free amino group at one end and a free carboxyl group at the other. Thus, for instance, the insulin submolecule of molecular weight 12,000 with four free α-amino groups contains four open polypeptide chains. It may be that other types of chains such as branched or cyclic chains also exist. Cyclic chains have been shown to be present in 'gramicidin S' (Sanger, 1946) and tyrocidine (Christensen, 1945), and the absence of a free amino group in ovalbumin suggests that this too may have a cyclic structure.

Table 6. *Terminal residues of proteins*

Protein	Assumed mol. wt.	Terminal residue: Amino-acid	Terminal residue: No. per mol.	No. of free ϵ-amino groups of lysine	No. of lysine residues per mol.	References
Insulin	12,000	Glycine	2	2	2	Sanger (1945)
		Phenylalanine	2	—	—	
Haemoglobins:						
Horse	66,000	Valine	6	40	39	Porter & Sanger (1948)
Donkey	66,000	Valine	6	41	—	
Human	66,000	Valine	5	43	—	
Cow	66,000	Valine	2	47	—	
		Methionine	2	—	—	
Sheep	66,000	Valine	2	47	45	
		Methionine	2	—	—	
Goat	66,000	Valine	2	48	—	
		Methionine	2	—	—	
Myoglobin (horse)	17,000	Glycine	1	20	19	Porter & Sanger (1948)
Edestin	300,000	Glycine	6	50	48	Sanger (unpublished)
		Leucine	1	—	—	
β-Lactoglobulin	40,000					
Native		Leucine	3	19	31	Porter (1948)
Denatured		Leucine	3	32	31	
Ovalbumin	44,000	None	—	19	20	Porter (unpublished)
γ-Globulin (rabbit, native)	170,000	Alanine	1	65	95	Porter (unpublished)
Salmine	6,000	Proline	?	0	0	Porter & Sanger (1948)

In column 5 of Table 6 are shown the numbers of free ϵ-amino groups of the lysine residues. These were estimated by determining the amount of ϵ-DNP-lysine in a hydrolysate of the DNP-protein. In all the proteins studied except β-lactoglobulin and certain serum globulins it was found that the total number of free ϵ-amino groups of lysine was equal to the total lysine content of the protein (shown in column 6). In β-lactoglobulin, however, all the ϵ-amino groups were free only after denaturation (Porter, 1948), thus indicating that certain of the amino groups are unreactive in the native protein in the same way that certain —SH and phenolic —OH groups are unreactive in some native proteins.

This method can also be used to obtain information about the course

of hydrolysis of proteins. Thus Desnuelle & Casal (1948) have demonstrated the great lability to concentrated HCl of peptide bonds involving the amino groups of serine and threonine. They have obtained evidence that a shift of the bond takes place from the amino group to the hydroxyl group under the influence of the strong acid.

The DNP technique can also be carried further and be used for the separation of peptides (Sanger, 1948). Thus in a partial hydrolysate of the DNP-derivatives of certain fractions of oxidized insulin the following peptides and their intermediate breakdown products were detected:

DNP-glycyl-isoleucyl-valyl-glutamic acid,
DNP-phenylalanyl-valyl-aspartyl-glutamic acid,

and their structure was worked out by subsequent partial hydrolysis. Peptides of this type could be very satisfactorily fractionated on silica, and buffered columns were useful for separating the less soluble peptides. One advantage of this method is that the final peptide mixture is relatively simple, so that it is possible to separate somewhat larger peptides than by other methods, but it only deals with a limited part of the molecule which is near the free amino groups. Woolley (1948) has also reported the separation of a number of DNP-peptides from a tryptic digest of DNP-insulin by Craig's method of distribution analysis, which employs the same principle as partition chromatography.

CONCLUSIONS

From the above review it can be seen that the fine resolving power of partition chromatographic methods has opened up new fields in protein chemistry, but that the contributions of the method have only just started.

Paper chromatography has given us an almost ideal method for qualitative amino-acid analysis, and it seems likely that a similar method may prove to be the basis for an ideal quantitative analysis. Paper chromatography has been used with great success for the separation of small peptides and for determining their structure. More dipeptides have been detected by the Leeds workers using this method than have previously been identified by the classical methods of organic chemistry. Whether the complete structure of a protein could be worked out from such small peptides is doubtful, and we are still in need of a general method for the fractionation of higher peptides. It may be that partition chromatography will supply this, but they are not easily fractionated on the usual silica, paper or starch chromatograms.

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CONTRIBUTIONS TO THE DISCUSSION OF ABOVE PAPER

Application of Paper Chromatography to Partially Hydrolysed Proteins.

By R. Consden. *Wool Industries Research Association, Leeds* 6

Paper chromatography has been employed to identify lower peptides in complex mixtures, usually after preliminary separations by other means, and hence to provide a knowledge of the order of amino-acid residues in polypeptides and proteins.

Of about twenty dipeptides of glutamic and aspartic acids identified in partial hydrolysates of wool, glutamylglutamic acid occurred in highest amount, showing that a relatively high proportion of the glutamic acid or glutamine residues are linked to similar residues in the wool protein. The results also show that the dicarboxylic amino-acids and/or their amides are linked both through carboxyl and amino groups with residues of most of the neutral amino-acids. Similar studies have been carried out of the peptides of cystine, after their oxidation to peptides of cysteic acid. Some twelve such dipeptides have been identified, showing that, as in the case of the acidic amino-acids, cystine is linked both through carboxyl and amino groups with residues of most of the neutral amino-acids. In spite of the failure to identify a dipeptide consisting of proline and cysteic acid residues, evidence for the occurrence of a relatively high proportion of proline residues next to, or near to, residues of cystine is provided by the fact that the tri- and higher peptides of cysteic acid gave much proline on hydrolysis; this would suggest that the polypeptide chain of wool is bent at right angles near the disulphide linkages.

Since the lability of peptide linkages varies considerably, according to the constituent amino-acid residues, it does not follow that the absence of any given peptide in a hydrolysate necessarily implies the absence of this sequence in the original structure, unless this peptide can be shown to occur in another protein, containing similar amounts of these amino-acids, and hydrolysed under similar conditions. It seems desirable that various conditions of hydrolysis should be investigated, as it might be possible to find conditions in which labile linkages are relatively more stable. In any case, existing knowledge of the kinetics of hydrolysis of peptides needs to be greatly extended in order to provide a sound basis for interpretations of results of partial hydrolysis studies.

Radioactive Isotopes in Paper Chromatography.

By J. E. Page. *Research Division, Glaxo Laboratories Ltd., Greenford, Middlesex*

Radioactive isotopes could be used for the quantitative estimation of amino acids on paper chromatograms. In the technique described by Keston, Udenfriend & Levy (1947), the isotopic *p*-I^{131}-phenylsulphonyl (pipsyl) derivatives are separated on a paper chromatogram, and successive 5 mm. strips of the chromatogram are counted under a Geiger-Müller counter. Keston *et al.* used the technique to examine silk hydrolysates. By using a second isotope such as S^{35}, the estimation can be made independent of complete resolution of the bands.

Dr Page thought that the Geiger-Müller counting method (cf. also Tomarelli & Florey, 1948), and the related radioautograph techniques (Fink, Dent & Fink, 1948; Fink & Fink, 1948), showed great promise, and would be more widely used in this country when biochemists had had more experience with radioactive isotopes. Such quantitative techniques might supersede those in which the amino-acids were eluted from the paper chromatogram before estimation.

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4. APPLICATIONS TO STUDY OF AMINO-ACID AND PROTEIN METABOLISM

By C. E. DENT

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Partition chromatography has not yet been applied to metabolic studies to the same extent as to problems of protein structure. Moreover, only filter-paper methods have been used up to now, presumably because they are the most useful for the first rough survey of the problem at hand. Column methods are likely to become more necessary later on when bulk isolations of materials are required. This review has therefore to be somewhat sketchy, and it will introduce speculative matter and such uncompleted work as may help, if nothing else, to illustrate the scope of paper chromatographic methods. The writer's conviction is that in paper chromatography we have a most specific and reliable method for rapid qualitative and rough quantitative analysis of mixtures of amino-acids and other readily water-soluble compounds. By its use a comprehensive study of the free amino-acids in tissue fluids can now be carried out. This little-known field is surely more likely, in the present state of our knowledge, to yield useful information about protein metabolism than is any study necessarily limited as heretofore to protein materials extracted from tissues.

The first fact likely to be noticed by an investigator of non-protein-containing biological fluids is the existence of very many (often 20–30) substances which move characteristically on the paper chromatograms and which give typical purple colours on treating in the usual way with ninhydrin. Most, but usually not all, of these can be identified as the amino-acids commonly found in protein hydrolysates. The few exceptions pose a difficult problem. In order to help in their identification the writer has collected and studied on the chromatograms as many substances as possible which could be expected to occur in such fluids. A 'Map of the Spots' has now been prepared showing the positions occupied by some sixty amino-acids or closely related compounds (Dent, 1948), and further compounds are being added to the map all the time. This has already enabled some of the 'unknowns' to be provisionally identified. Of these the more interesting are; α-amino-*n*-butyric acid, γ-aminobutyric acid, methionine sulphoxide and methyl-histidine. In addition, monoiodotyrosine has been found in rats' thyroid gland (Fink & Fink, 1948) and α-γ-diaminobutyric acid has been found in the antibiotic aerosporin (Catch & Jones, 1948); this latter identification,

unlike the others, has been confirmed by isolation and ultimate analysis. It is clear that future studies of amino-acid metabolism will have to take account of the existence of these compounds. 'Unknowns' are often found that are unstable to acid hydrolysis. These are likely to be peptides or else amino-acids substituted elsewhere than on an amino group, e.g. amino-acid amides or *o*-phosphoryl derivatives. Identification of these necessitates prior isolation by the cutting out technique (Consden, Gordon & Martin, 1947; Dent, 1947*a*). It should be noted that primary aliphatic amines, unless they are too volatile to stay on the paper, can be identified at the same time as the amino-acids, since they give a similar ninhydrin colour reaction.

A few words follow about the possible snags in the identification of compounds by paper chromatography. First, too much reliance should not be placed on the results obtained by the one-dimensional method. This is because of the frequent occurrence of overlapping, different substances moving very often at the same speed in the one solvent used. Two-dimensional analysis, however, is much more specific. Secondly, no substance should be identified unless it has been proved to be stable to acid hydrolysis. Omission of this precaution will risk confusion between an amino-acid and the almost infinite number of peptides and other like complexes. Thirdly, the accuracy of the position matching on the chromatogram must be checked by running the unknown substance both when mixed with the pure compound which it is believed to be and again with other amino-acids which run to neighbouring positions. The latter enables its relative position to be ascertained accurately without needing to place any reliance on the R_F values, which are less reproducible. When such reasonable precautions are taken the only likelihood of wrong identification will be in the case of a totally unrelated compound, not expected to be present, which happens to behave similarly to a known substance. This has occurred already in the writer's experience in the case of citrulline, which was for a time confused with β-alanine. This risk is, however, small, and it decreases as our knowledge of natural substances increases.

TECHNIQUE

Only minor modifications of the original method of Consden *et al.* (1944) have been reported (Dent, 1947*a*, 1948; Williams & Kirby, 1948). For testing urines (as distinct from tissue fluids of more constant concentrations) for the presence of a pathological output of amino-acids, the writer now prefers to take a constant amount of total nitrogen (i.e. 250 μg.) for the analysis, rather than a constant volume (25 μl.) as previously recommended (Dent, 1946). The constant volume remains

convenient as a rough test and is reliable enough in the absence of polyuria.

As applied in this way to the untreated urine or other fluid, the method is inadequate for studying the amino-acids present only in traces, since the total volume taken cannot be increased indefinitely without interference from the inorganic salts always present. The ions of the latter also move characteristically during the development of the chromatogram (Westall, 1948; Consden & Gordon, 1948) and finally occupy large areas of the paper. Glutamine, and aspartic and glutamic acids may occupy their normal positions and overlie the inorganic ions, but usually they are 'shouldered off' by the salts and finally appear as distorted streaks surrounding parts of the salt areas. This makes these three amino-acids more difficult to recognize, and it may be impossible to evaluate roughly the amount present from the colour strength. This problem can now be circumvented by prior removal of most of the salts by the ingenious desalting device of Consden *et al.* (1947), which can be readily adapted to deal with 1–10 ml. of fluid. After desalting in this way, over 3 ml. of, for instance, cerebrospinal fluid has been analysed satisfactorily on one chromatogram, whereas the original fluid showed slight distortions when only 0·1 ml. was used. This now enables many amino-acids to be detected at concentrations of only 1 μg./ml., and there is scope for increasing the sensitivity still further.

The writer is now experimenting with a method for distinguishing α-amino-acids from the many other substances which react similarly with ninhydrin. The original mixture is treated with the copper phosphate suspension of Pope & Stevens (1939) and the filtrate analysed on chromatograms as usual, taking care during the phenol run not to add the cyanide or any other substances which would decompose the copper complexes. The α-amino-acids form copper complexes under these conditions which run very fast in the solvents and only give very faint, if any, ninhydrin colours. Substances such as primary amines, γ-, δ-, and ϵ-amino-acids survive this treatment and appear as usual in their appointed places. The behaviour of β-alanine and of other β-amino-acids in this test has not been properly studied yet. β-Alanine appears to form a copper complex grudgingly (Woiwod, 1948).

The great possibilities inherent in paper chromatography when used in combination with radioactive materials are now beginning to be drawn upon. Fink, Dent & Fink (1947) have shown that radioactive iodine when taken up by the thyroid gland of the rat is held in the form of inorganic iodide, diiodotyrosine and at least three other unidentified substances, one of which is probably monoiodotyrosine. It was only necessary to press the chromatograms against an X-ray plate for a suitable time for the position of the radioactive spots to be located.

They could be identified by developing visibly in the usual manner marker substances added to the original mixture before chromatographic analysis. Working in a similar manner Borsook, Deasy, Haagen-Smit, Keighley & Lowy (1948) have claimed that lysine can be converted into α-aminoadipic acid by a liver homogenate. Stepka, Benson & Calvin (1948) are also tracing on paper chromatograms the radioactive amino-acids formed when green algae are exposed to $C^{14}O_2$ for 30 sec.

AMINO-ACID METABOLISM

The original method of approach to the problem of the metabolism of individual amino-acids is exemplified by an experiment of Van Slyke & Meyer (1913). In this, glycine was fed to dogs, and at various intervals the dogs were killed and samples of blood, urine and of the tissues were analysed. The increase of amino-nitrogen above normal was attributed entirely to the effect of additional glycine, for which no specific method of analysis was available.

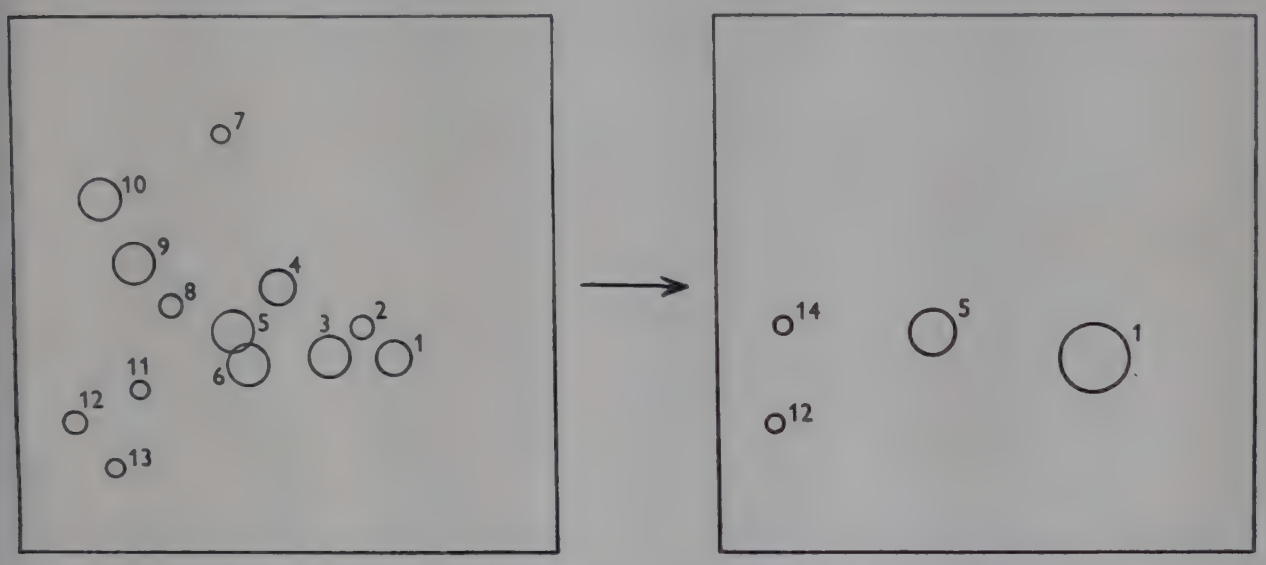

Fig. 1. Diagrams of chromatograms from 125 μl. of dog's portal blood ultrafiltrate, before and 1 hr. after giving glutamic acid by mouth. The sample has been placed at the bottom right-hand corner of the paper. Phenol has been run from right to left as first solvent, 'collidine' upwards as second solvent. The convention is used of representing the strength of the ninhydrin colour reaction by means of the size of the spot, using an arbitrary scale. 1, glutamic acid; 2, serine; 3, glycine; 4, threonine; 5, alanine; 6, glutamine; 7, tyrosine; 8, α-aminobutyric acid; 9, valine; 10, leucine and/or isoleucine; 11, γ-aminobutyric acid; 12, arginine; 13, lysine; 14, proline.

More recent work has shown that this method is not of general validity, as it ignores the possible effects of large amounts of one amino-acid on the concentrations of the others, all of which contribute to the total amino-nitrogen. For instance, after feeding glutamic acid only a small change, if any, takes place in plasma amino-nitrogen concentration (Seth & Luck, 1925). It is now known that the plasma glutamic acid level rises appreciably in such a case, but that the effect of this

Table 1. *Metabolism of single amino-acids given in relatively large amounts*

Amino-acid	Amount and method of administration	Animal used	No. of exps.	Blood changes (125 μl. taken)	Urine changes (25 μl. taken)
Glycine	Diet comprised 10% of glycine as sole source of N. Administered for about 1 month before tests done	Rat	2	—	Very large increase in concentration of glycine. Less large increase in serine concentration
DL-Serine	5 g. given by mouth 3 hr. before tests	Patient with Fanconi syndrome	1	—	Very large increase in concentration of serine
DL-Serine	100 mg. given by stomach tube daily for 6 days before test	Rat	1	—	Large increase in serine concentration
L-Cystine	8 g. given by mouth	Patient with Fanconi syndrome	1	—	No change in amino-acid pattern
L-Aspartic acid (as Na salt)	350 mg. given by stomach tube	Rat	2	—	Very large increase in concentrations of aspartic and glutamic acids
L-Glutamic acid	55 g. sodium salt by mouth (3 g./kg.)	Dog	1	Very large increase in glutamic acid. Alanine, proline and arginine in about normal quantities. All other amino-acids undetectable	Very large increase in glutamic acid concentrations. Slight increase in aspartic acid
L-Lysine hydrochloride	400 mg. by stomach tube	Rat	2	—	Large increase in concentrations of glutamic acid and of taurine, slight increase in lysine. No α-aminoadipic acid seen

DL-Methionine	10 g. given by mouth	Patient with Fanconi syndrome	2	—	Large output of methionine and of methionine sulphoxide. Definite but less marked increase in α-aminobutyric acid
DL-Methionine	10 g. given by mouth	Normal human	2	—	Large increase in concentration of methionine and of methionine sulphoxide. Traces of α-aminobutyric acid. No other amino-acids seen
DL-Methionine	10 g. given by mouth	Dog	1	Large increase in concentration of methionine and of methionine sulphoxide.* Slight increase in α-aminobutyric acid	—
DL-Isoleucine	Given by mouth as supplement for isoleucine-deficient diet	Normal human baby	1	—	Large increase in concentration of isoleucine. Other amino-acids in normal concentrations

* In this chromatographic analysis the identification of the sulphoxide is doubtful, as the precaution was not taken of making sure it did not arise from methionine by aerial oxidation during the analysis.

N.B. The aspartic acid and lysine experiments were conducted in collaboration with Dr Neuberger, the glutamic acid with Dr J. Schilling and the isoleucine experiment with Drs L. E. Holt and A. A. Albanese.

on the total amino-nitrogen is largely offset by a coincident fall in the concentrations of most of the other amino-acids (Christensen, Streicher & Elbinger, 1948). This change is illustrated in Fig. 1. Similar changes are also reported after injection of leucine, isoleucine and methionine (Hier, 1947). Such studies should therefore cover all these possible complications, and since methods for determining all the amino-acids in blood do not exist, and since unidentified amino-acids can also occur, it follows that paper chromatography is likely to be of very great value, at least as a preliminary method of surveying the blood or urine changes after feeding individual amino-acids. A few of the writer's results which illustrate these points are given in Table 1.

Similar principles are involved in the study of the metabolism of mixtures of amino-acids, whether synthetic or present as protein hydrolysates. Dent & Schilling (1948) have studied on paper chromatograms the effect of an ingestion of Amigen (casein hydrolysate) by dogs. The patterns were compared of the original hydrolysate, of the portal and jugular plasmas, and of the urine. The ingestion of a badly balanced mixture of amino-acids was not investigated. It seems likely that it would show characteristic blood changes, which might indicate which amino-acids are required to be added to the mixture so as to make it more readily taken up by the tissues.

The action of hormones which affect nitrogen metabolism (e.g. testosterone, corticosterone, pituitary growth hormone, insulin) on the blood amino-acids is open now to more detailed investigation.

PROTEIN METABOLISM

The problem of the digestion, absorption and utilization of protein is somewhat similar to that of the amino-acid mixtures as discussed above. However, two new points arise which may be of importance. First, that a fed protein may not be completely broken down in the gut but may be absorbed, at least in part, as peptides, some of which may have special physiological activity (Woolley, 1946*a*). Secondly, that the amino-acids are liberated at different rates, and sometimes incompletely, during enzymic digestion in the gut, and are not therefore being simultaneously presented to the gut wall and thence to the tissues as must happen when the amino-acid mixture has been given intravenously or when it has been taken by mouth.

It is probably for this reason that differences have sometimes been found between the feeding of intact protein and of an apparently equivalent amino-acid mixture. As a result, the amino-acid analysis of a protein may not be the sole criterion of its nutritional value, which latter then becomes most difficult to evaluate (Block & Mitchell, 1946).

Digestion and absorption of proteins from the gut have been studied in a preliminary manner by Dent & Schilling (1949). Using the dog as experimental animal they followed the amino-acid changes in portal and jugular plasma ultrafiltrate after the ingestion of casein, ground beef and human-serum albumin. The blood samples were taken before the experiment began and 1, $2\frac{1}{2}$ and 5 hr. after. Most of the dogs had a partial gastrectomy operation previously performed on them. This resulted in much quicker disappearance of the administered protein from the gut, and very large rises—up to sixfold—in the total amino-nitrogen concentration of the plasma ensued. The detailed changes could be readily followed on paper chromatograms. The large increase in amino-nitrogen almost entirely concerned the common amino-acids. No 'peptides', i.e. spots on the chromatograms in unusual places and which were unstable to acid hydrolysis, were found in the blood, although small amounts of 'bound amino-nitrogen' of unknown origin were sometimes found, as shown by an increase in the concentration of some amino-acids after acid hydrolysis of the sample. Many of these results were checked by Christensen (1949), who determined free α-amino-nitrogen before and after hydrolysis by the very specific ninhydrin/CO_2 method. The serial blood changes after giving casein were very similar to those after casein hydrolysate. In the latter case absorption into the portal blood as free amino-acids can be safely assumed to be at least the predominant mechanism. It was remarkable that the rate of absorption of the hydrolysate, as measured by the blood changes, was only slightly faster than that of the intact protein.

Dent & Schilling concluded that the main absorption of the protein was in the form of free amino-acids. The possibility of small amounts of peptide absorption was not, however, excluded, especially since it could be suggested that peptides may be more rapidly removed from the blood by the liver so that they would only appear in the portal blood. The concentrations in such a case could be quite small, since the quantities could not accumulate as the amino-acids undoubtedly do during repeated circulation in the vascular system. However, they believed that in these experiments peptide absorption could only have paid at most an unimportant contribution to the mechanism by which protein nitrogen was absorbed into the blood stream. Only rarely did Christensen find a rise above normal of the portal concentration of 'bound amino-nitrogen' of more than 2 mg./100 ml. If reasonable assumptions are made as to the likely portal blood flow we can calculate that had this peak concentration been maintained for the 5 hr. span of the experiment, and if we assume that it were all present as peptides, one could only account for the absorption from the gut of about 2 g. of the 15 g. of nitrogen fed in the form of protein. No evidence was found

in these experiments of any appreciable differences in the rates of absorption from the gut of different amino-acids. It must be stressed, however, that no vegetable, or known incomplete proteins, were among those investigated. It would be interesting to study by this method the anomalies believed to occur in the absorption of, for example, heated casein (Pader, Melnick & Oser, 1948) or raw soya-bean meal (McGinnis & Evans, 1947).

In marked contrast to the results on the above proteins, no appreciable changes in blood amino-acid or bound amino-nitrogen levels were observed in three experiments in which the same quantity (100 g.) of dog's whole plasma protein was given by mouth. Indeed, there was a tendency for the amino-nitrogen to fall during the digestion and absorption, a fact that the writer has since found to occur also in man after giving homologous plasma both by mouth and by vein. The tentative suggestion was made that such homologous plasma could be absorbed from the gut in intact form. There are considerable difficulties in accepting this however, since plasma so absorbed should be equivalent in every way to plasma given by intravenous injection. However, from the point of view of the nitrogen balance it has been shown in dogs (Holman, Mahoney & Whipple, 1934) and in man (Albright, Forbes & Reifenstein, 1946) that homologous plasma given by mouth has a completely different effect than when it is given by vein. In the former case the nitrogen administered appeared in the urine in a day or so, in the latter case only about one-third of it appeared in the next few days, and some was still unaccounted for after 3 weeks.

AMINO-ACID PROBLEMS IN THE NORMAL HUMAN

No systematic data are published on the normal levels of the different amino-acids in the various body fluids, as determined after a prior separation by paper chromatography. This is long overdue. Quantitative methods are now available (Woiwod, 1948), although for many medical purposes the rough estimation, against standards, of the intensity of the ninhydrin colour reaction is adequate.

The high outputs of methionine, 250–500 mg. per day, in normal human urine, claimed by Albanese, Frankston & Irby (1944) on the basis of an H_2O_2 oxidation method, have been disputed by Tomich (1947) who used a chromatographic test.

It is interesting to compare normal cerebrospinal fluid (C.S.F.), plasma and urine. The chromatograms show that in C.S.F. the amino-acids are all much weaker than in plasma, except for glutamine, which occurs at about the same concentration. This is eloquent additional proof that the C.S.F. cannot be a simple ultrafiltrate of plasma. Much more marked differences occur between the patterns of amino-acids in plasma and

urine. Roughly speaking, the plasma amino-acids comprise mainly the 'essential' higher molecular weight members. In urine it is the rule for the 'unessential' lower molecular weight amino-acids (glycine, alanine, etc.) to predominate. The kidney threshold level appears therefore to be much higher for the more essential substances, as if the body retained them on a teleological basis. Hydrolysis of urine, before running on the chromatograms, usually results in slight strengthening of many of the amino-acid spots; glutamic and aspartic acids, and sometimes glycine, however, nearly always increase to an exceptional extent. There appear therefore to be large amounts of hydrolysable complexes of glutamic and aspartic acids in the urine. As these do not give the ninhydrin reaction they are presumably not simple peptides, but are probably *N*-substituted acyl derivatives of the type of hippuric acid.

DISORDERS OF AMINO-ACID OR OF PROTEIN METABOLISM

At least five human diseases exist the nature of which suggests the possibility of some error of amino-acid or of protein metabolism. These will be considered below in turn. They have not yet been exhaustively studied by chromatographic methods. Preliminary work on other forms of liver disease (Dent, 1947*b*; Young & Homburger, 1947), on progressive muscular atrophy (Ames & Risley, 1947) and on hepato-lenticular degeneration (Uzman & Denny-Brown, 1948) has also been reported.

(1) *Acute yellow atrophy of the liver*

The well-known rise in the amino-acid content of blood and urine in this disease can be readily confirmed by paper chromatography. The earlier work was based on actual isolations of some of the less water-soluble amino-acids, leucine and tyrosine in particular, or on total amino-nitrogen determinations, and it was not until the chromatographic method became available that it could be said with certainty which other amino-acids were also involved. The writer has studied five cases so far that would be clinically definable as the classical 'Acute yellow atrophy', i.e. a rapidly fatal form of jaundice, occurring without previous liver damage, and showing the histological picture of massive hepatic necrosis (Himsworth, 1947). In all cases an increased urinary concentration of amino-acids was found which involved nearly all the commonly known members, and in the one case showing the highest plasma level of α-amino-nitrogen (37 mg./100 ml.). Evidence of the presence of peptides was also found (Dent, 1947*b*). Unexpected differences in amino-acid pattern between the blood and urine occurred, but no particular significance has been drawn from this yet. Of especial interest was the almost constant finding of substantial rises in the blood

levels of methionine and cystine. This could be submitted as evidence against the theory that acute yellow atrophy is likely to be curable by giving additional amounts of these substances.

Less serious forms of liver disease in man, however, do not appear to result in any marked change in amino-acid metabolism. The view long held that 80% or more of the liver had to be destroyed before any disordered amino-acid metabolism could be detected, appears to be supported by these pathological studies. The practical outcome is therefore that the chromatographic test for urinary excretion (Dent, 1946) is quite useless to detect mild liver damage, which condition is unfortunately where a test is most needed at the moment. As a test for very serious liver damage, however, it does remain of value, especially as an aid to prognosis. So far patients with gross amino-aciduria resulting from liver disease have, with one exception, all died quickly. The exception was a case of jaundice of unknown etiology, associated with urticaria and other unusual features. The amino-aciduria only lasted about 1 day, during most of which the patient was unconscious and also had an epileptic fit. Complete recovery resulted within 2 weeks. In one of the cases of acute yellow atrophy the chromatographic test was strongly positive before a clinical diagnosis could be made with certainty, so that it seems likely that it would have been positive some days earlier, when there was nothing to distinguish it from an ordinary mild case of infectious hepatitis.

Rats with acute hepatic necrosis produced by dietary means (Glynn, Himsworth & Neuberger, 1945) have also been found to show amino-aciduria and occasional peptiduria (Dent, 1947*b*).

(2) *The Fanconi syndrome*

A study of the urine in three cases of this disease has already been reported (Dent, 1947*a*). The suggestion was originally made by Fanconi (1936), on indirect evidence, that there was a large output of amino-acids in the urine. This is now fully confirmed by paper chromatography and by chemical means, and the writer has now been able to study the urines in a further nine cases.

The Fanconi syndrome is essentially a form of rickets or osteomalacia which is resistant to ordinary doses of vitamin D and in which among other things amino-aciduria and renal glycosuria also occur. It shows not the least clinical resemblance to acute yellow atrophy, so it is not surprising to find that the very similar urinary changes, with respect to the amino-acid concentration, are brought about by an entirely different mechanism, namely, by a low renal threshold for amino-acids, the blood level of amino-nitrogen remaining normal. The urinary output of amino-nitrogen may rise to about 15% of the total nitrogen, and the pattern

of excretion of the different amino-acids, as seen on the chromatograms, may be quite different from case to case. This is illustrated in Fig. 2, where the chromatograms from four urines are shown diagrammatically. Presumably the kidney tubular disfunction responsible for the failure to reabsorb adequately the amino-acids in question varies slightly in

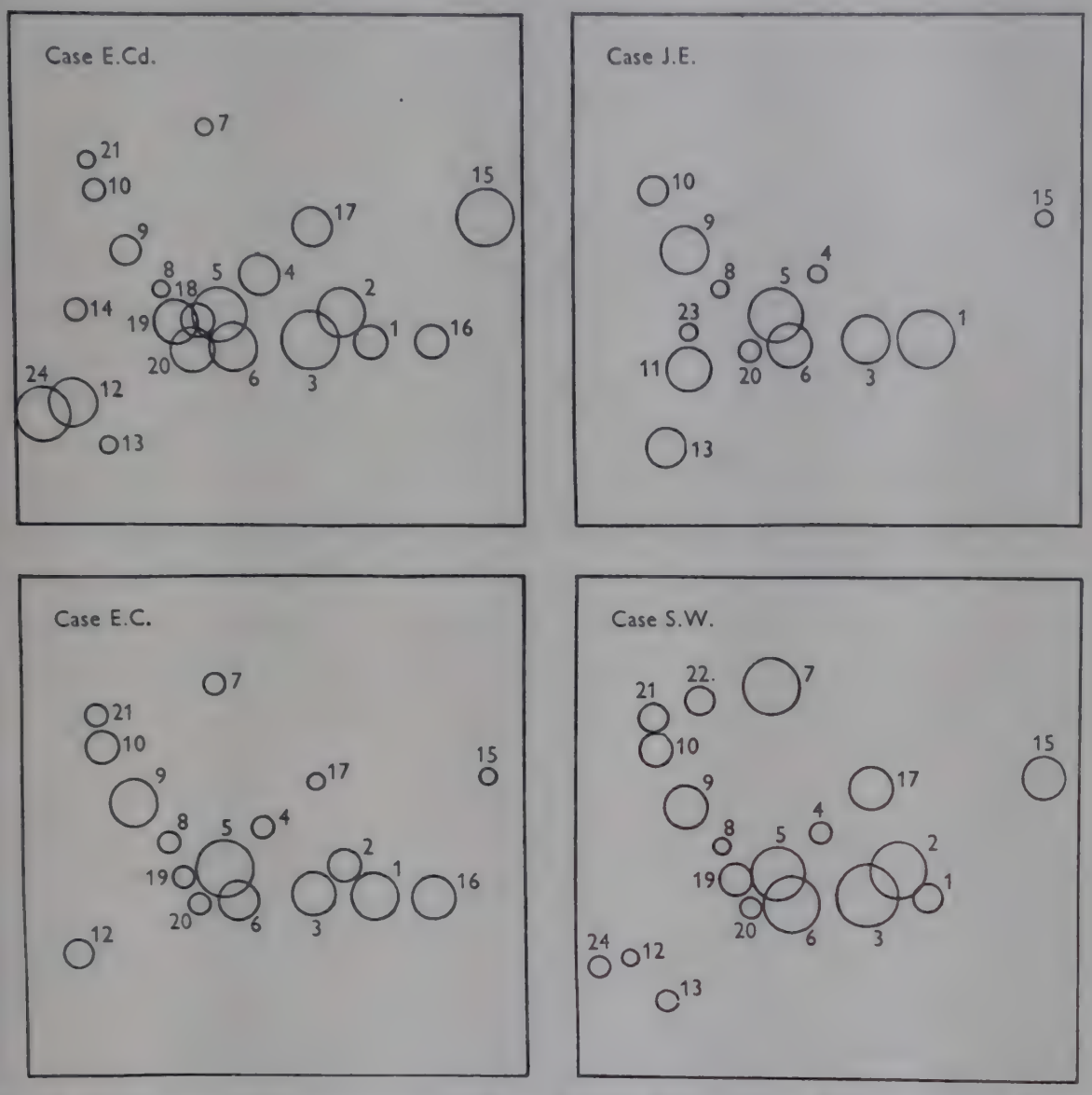

Fig. 2. Diagrams of chromatograms from 25 μl. of the urine of four cases of Fanconi syndrome. They were specially chosen to illustrate the very different patterns that may be found in this disease. The same conventions are used as in Fig. 1. Owing to the method of representation the overlaps between neighbouring spots are grossly exaggerated here. 15, cysteic acid (from cystine); 16, aspartic acid; 17, taurine; 18, hydroxyproline; 19, histidine; 20, citrulline and/or β-alanine; 21, phenylalanine; 22, tryptophane; 23, methionine sulphoxide (probably); 24, 'fast-arginine' —an unidentified substance.

its location from case to case. In a given case, however, the amino-acid pattern appears to be quite characteristic. For instance, E.C. (Fig. 2) was followed for 7 months, during which over forty two-dimensional chromatograms were performed on different 24 hr. urine samples. The patterns were quite constant as far as could be judged by the method used except after giving large amounts of individual amino-acids by

mouth. The same individual pattern was also obtained from two samples of the urine of E.Cd. taken 1 year apart, and from two samples of the urine of S.W. taken 4 months apart. This variation in pattern in different cases of the disease as well as the 'renal' mechanism of its production is strong evidence against the presence of any true error of metabolism. It also makes it difficult to attribute any harmful effect to the amino-aciduria, since the dietary intake of any individual amino-acid (in the form of protein) is usually several times larger than the loss in the urine.

In one of the three first cases studied an unusual spot ('under-alanine') appeared on the chromatogram close to the position occupied by glutamine. Attempts to isolate and identify the substance by the cutting out technique suggested that it was a peptide of serine and glycine, possibly seryl-glycyl-glycine. This is an interesting possibility, occurring as it did in a disease showing defective bone growth, since Woolley (1946*b*) has found that certain serine peptides such as seryl-glycyl-glutamic acid may have growth-promoting properties. Unfortunately, the writer has not been able to find 'under-alanine' in the urine in any further case of the disease, so its significance does not appear to be at all clear. An exactly similar clinical type, i.e. Fanconi syndrome with cirrhosis of the liver and portal obstruction, has not been encountered again among the other cases studied.

(3) *Cystinuria*

Studies by Dent & Rose (unpublished work) of the urine in five cases of cystinuria has revealed that the preponderant amino-acid is often lysine, and that there are sometimes abnormal quantities of arginine, ornithine, taurine and of three other ninhydrin-reacting substances, as yet unidentified. One of the latter may be cadaverine and/or putrescine, which are already known to occur occasionally in cystinuric urine (Garrod, 1908); the others are probably uncommon amino-acids. Diagrams of the chromatograms from four of the urines appear in Fig. 3. The additional amino-acids occur together with the usual amounts of the amino-acids commonly found in normal urines (glycine, alanine, etc.).

These preliminary results make it difficult to accept the theory usually advanced that in cystinuria there is primarily an error of metabolism leading to overproduction of cystine or to an inability to metabolize it further. Such a theory cannot be readily reconciled with the occurrence in the urine in similar large quantities of other substances totally unrelated structurally to cystine, and of the minor variations found from case to case in the amino-acid pattern. The facts presented do, however, recall the findings in different cases of the Fanconi syndrome

(Fig. 2), and it is suggested therefore that a similar 'renal' mechanism may account for the urinary output of amino-acids in cystinuria, slight variations in the localization of the disfunction of tubular reabsorption accounting for the different urinary patterns. Clearly the blood must be examined and proved to be normal if this is to be upheld. This work

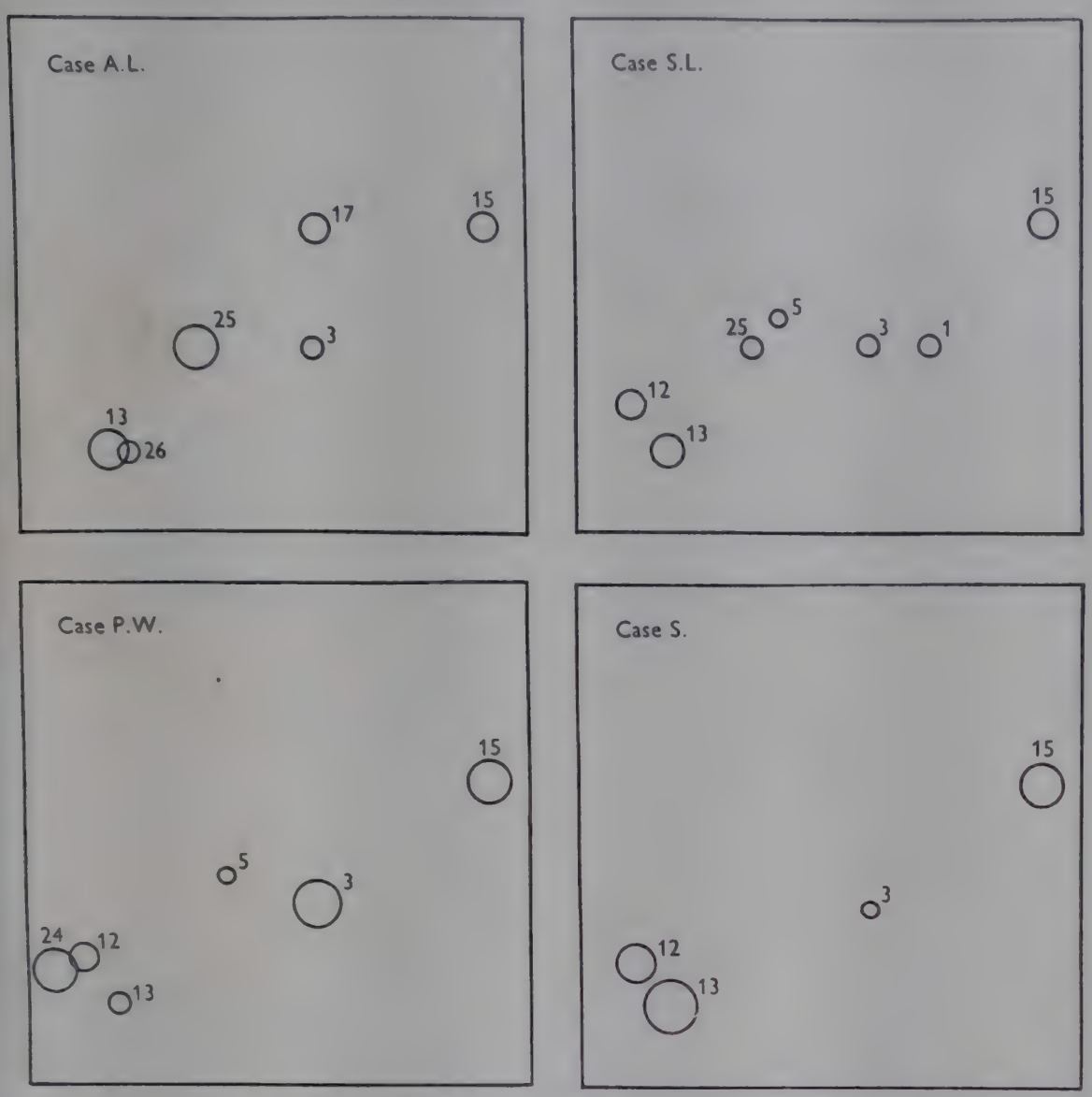

Fig. 3. Diagrams of chromatograms from 25 μl. of the urine of four cases of cystinuria. The same conventions are used as in Figs. 1 and 2. 25, 'On-citrulline'—an unknown substance moving very close to the area occupied by citrulline; 26, ornithine—this substance requires a separate chromatographic step to be performed in order to distinguish it from lysine. It has not yet been looked for in this way in the other three urines.

is in progress. Yeh, Frankl, Dunn, Parker, Hughes & Gyorgy (1947), using microbiological methods of analysis in the urine of one case of cystinuria, have also independently found an excessive excretion of arginine and lysine in addition to the cystine. They state, however, without going into details, that their findings can be explained by the theory of a general disturbance of amino-acid metabolism.

A 'renal' theory of cystinuria would explain readily its clinical harm-

lessness (unless stones form) and its non-conformity (Lewis, 1932) with the 'all-or-none'* character of true inborn metabolic errors such as alkaptonuria or albinism. It may also help to explain the puzzling effect of fed cystine and methionine on the urinary output of cystine. Brand, Cahill & Harris (1935) reported that methionine, but not cystine, given by mouth, increased the cystine output in a case of cystinuria. Brown & Lewis (1941), using rabbits, showed that after giving cystine by mouth there was no change in the plasma cystine level. However, there was a rise after giving methionine. This, if confirmed in man, would suit the 'renal' theory of cystinuria very well, as the urinary output of cystine would only be expected to rise subsequent to a procedure that resulted in a rise in the plasma concentration. However, a contradictory report is now in the literature. Hier (1947) has claimed that the plasma cystine level does not rise after either cystine or methionine by mouth.

(4) *Phenylketonuria (Phenylpyruvic oligophrenia)*

The evidence is now very strong that in this disease there is a true error of metabolism of a single amino-acid. Jervis (1947) has shown that patients with the disease cannot hydroxylate phenylalanine to turn it into tyrosine. This hydroxylation is probably the first stage in the breakdown of phenylalanine. Tyrosine itself can, however, be normally dealt with. Owing to the inability to metabolize phenylalanine this substance accumulates in C.S.F. (Jervis, Block, Bolling & Kanze, 1940), blood and urine. On passing through the kidney most of it is converted into phenylpyruvic acid. Jervis used chemical methods to determine the phenylalanine. Dr O. Lindan (unpublished work) has recently examined on paper chromatograms the C.S.F. from a patient with phenylketonuria, as well as from several normal people. The remarkable increase in phenylalanine concentration was readily confirmed, but there was no comparable abnormality noted in the pattern of the other amino-acids. The disorder found in the metabolism of only one amino-acid contrasts markedly with the relatively complicated changes in amino-acid concentrations found in cystinuria and in the Fanconi syndrome.

(5) *Multiple myelomatosis and Bence-Jones proteinuria*

Many theories have been advanced to explain the production by the tumour cells in this disease of an abnormal blood protein, which usually appears also in the urine under the name of Bence-Jones protein.

* It is conceded that the expression 'all-or-none' is not strictly accurate, and that the metabolic error in question can usually be expressed purely quantitatively. The quantities in question are, however, much larger than occur in other kinds of diseases.

Perhaps the most plausible one is that which assumes that an error of metabolism has occurred in the synthesis or degradation of a body protein, whereby this substance, which normally occurs only as a stage in some larger chemical process, cannot be further metabolized once it has been formed and so accumulates in abnormal quantities. If this were so, it would represent a unique example in the protein field of a derangement analogous to that occurring, with simpler molecules, in, for instance, alkaptonuria.

A chance discovery by paper chromatography that a pure sample of Bence-Jones protein, obtained from a typical case of myeloma, was methionine free has led to further investigations by Dent & Rose (1948). Two different chemical methods as well as biological and microbiological assays were later employed, and the results also confirmed the absence of methionine. Should this also be found for other samples of the protein, then it will be clear that the protein is most unusual in its chemical composition. This will afford strong evidence that it is quite different from the main fractions of the other plasma proteins so far analysed. Indeed, it would be likely to be either a protein quite foreign to the body or else present in amounts very much larger than normal. In their patient the urinary output of pure protein amounted to about 36 g. daily. The facts have led them to speculate that multiple myelomatosis may be caused by a virus infection, the Bence-Jones protein being the virus itself less its nucleic acid. If this were confirmed by more direct methods it would cause the disease to be removed from the category of errors of metabolism.

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CONTRIBUTION TO DISCUSSION OF ABOVE PAPER

An Application of Paper Partition Chromatography to Microbiology.

By Elizabeth Work. *Department of Chemical Pathology, University College Hospital Medical School*

The application of paper partition chromatography to microbiology is illustrated by an investigation on the amino-acids of *Corynebacterium diphtheriae*. The amino-acid contents of an alcoholic extract of the cells and of the insoluble cell residues were compared. The acid-hydrolysed cell residues contained eighteen amino-acids usually found in protein hydrolysates, together with one unknown ninhydrin-reacting substance, 'underglutamic acid', which is not a peptide. The alcoholic extracts showed, in addition to the above nineteen spots, spots coinciding with α-aminobutyric

acid, γ-aminobutyric acid, hydroxylysine, and an unknown basic substance which is not an α-amino-acid.

The appearance of α- and γ-aminobutyric acids in the soluble extracts of these micro-organisms but not in the cell proteins emphasizes the similarity between the protein metabolism of micro-organisms, plants and higher animals, since Dent found these amino-acids in plant extracts and in animal fluids such as blood and urine.

Rough estimations were made of the content in amino-acids of acid hydrolysates of insoluble cell residues of *C. diphtheriae* grown on different media. No significant differences were found, even between cells grown on media causing either large amounts of toxin to be produced, or none at all.

Cultivation of *C. diphtheriae* on a synthetic medium of known composition resulted in poor growth. The changes in the amino-acids of the medium caused by growth of the organism were followed by comparing chromatograms from equal volumes of the medium before and after growth. As expected, all the amino-acids showed a general lowering in concentration after growth of the organism, but aspartic acid and above all glutamic acid showed marked drops in concentration. Alanine appeared in the culture filtrate, although it was not originally present in the medium.

Following this result, the basal medium was changed and better growth of the organism resulted. In the culture filtrate the drop in glutamic acid was even more striking than in the first case; glycine but not aspartic acid showed a drop in concentration. Alanine, originally present in the basal medium, showed a fall in concentration, indicating that in this case it was being assimilated by the cells.

The disappearance of such large amounts of glutamic acid cannot be accounted for by its presence in the filtrate as glutamine or other hydrolysable derivatives. This was shown by acid hydrolysis of the culture filtrate which produced little difference in the strength of the glutamic acid spot.

Although this study of the changes in the medium occurring as a result of bacterial growth has produced practical results in the form of improved growth, the significance of the changes is not known. Much work remains to be done on the subject.

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5. PARTITION CHROMATOGRAPHY AND ITS APPLICATION TO CARBOHYDRATE STUDIES

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In the past fifteen years a number of attempts have been made to carry out the separation of sugars and sugar derivatives using chromatographic methods. Before the advent of partition chromatography these were based on selective adsorption and more or less followed the original technique of M. S. Tsvet which was developed more than 40 years ago. One of the most successful of methods on these lines was by W. S. Reich (1939), who separated coloured azoyl esters of glucose and fructose on a silica column by development with benzene-petroleum mixture. The usefulness of adsorption chromatography when applied to mixtures of sugars was rather limited by the fact that the procedures were very empirical. However, the introduction of partition chromatography by Martin & Synge (1941) and its further development by Consden, Gordon & Martin (1944) to the separation of free amino-acids on filter-paper chromatograms opened a field for the application of similar principles to the chromatographic separation of a fairly wide range of substances other than the amino-acids. Thus simple sugars and oligosaccharides of low molecular weight were separated on filter-paper by irrigation with solvents such as phenol-water, collidine-water or butanol-acetic acid-water (Partridge, 1946, 1948*a*). The reaction of the reducing sugars with a solution of silver nitrate in ammonia was used as a means of revealing the position of the sugars on the chromatograms, and other reagents were also developed for this purpose, particularly the use of a solution of naphthoresorcinol in trichloroacetic acid as a selective reagent for sucrose, raffinose and simple ketoses.

Fig. 1 shows the dispersion of R_F values of a number of sugars in two solvents, phenol-water and collidine-water, using Whatman no. 1 filter-paper. The R_F values in phenol have been plotted as ordinates and the values in collidine as abscissae. The figure shows that most of the sugars may be distinguished by application of these two solvents in the form of a 'two-dimensional chromatogram', but since in practice we usually have to deal with simple mixtures of a few sugars only, it is usually preferable to carry out a series of one-dimensional chromatograms, each in a different solvent. Experience shows that replicate chromatograms carried out with *at least three* different solvents are

necessary for the unambiguous identification of most of the common monosaccharides, and, in addition, to complete the identification, further replicate chromatograms should be sprayed with the various specific reagents that have now been developed for the purpose. Since

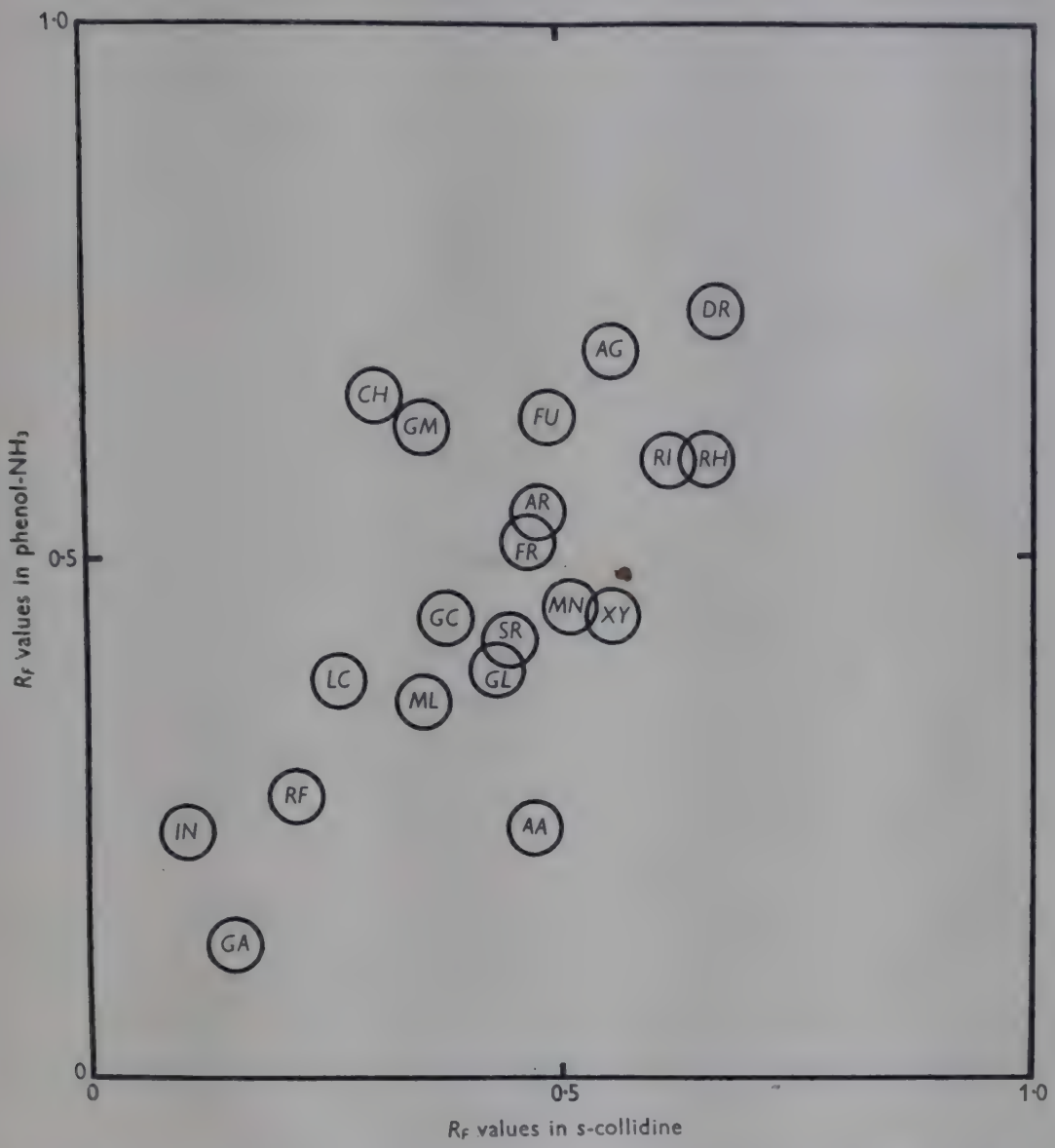


Fig. 1. Illustrating the separation obtainable by use of two solvents. The R_F values in phenol-1 % NH_3 and *s*-collidine are plotted at right angles. *AA*, ascorbic acid; *AG*, acetylglucosamine; *AR*, arabinose; *CH*, chondrosamine; *DR*, deoxyribose; *FR*, fructose; *FU*, fucose; *GA*, galacturonic acid; *GC*, galactose; *GL*, glucose; *GM*, glucosamine; *IN*, inositol; *LC*, lactose; *ML*, maltose; *MN*, mannose; *RF*, raffinose; *RH*, rhamnose; *RI*, ribose; *SR*, sorbose; *XY*, xylose.

the absolute R_F values are subject to many experimental factors difficult to control (even with non-ionizing solutes such as the sugars), the measurement of R_F values should not be relied upon for the purpose of identification, but in each case reference sugars should be included in the chromatogram, side by side with the unknown mixture.

INTERFERENCE

Interfering effects are of three main types. The first type is due to substances, other than the sugars, which react with the spraying reagent and give rise to coloured spots near those of the sugars. Thus the polyphenols and substances of a tannin-like character react with ammoniacal silver nitrate to give black or brown spots that are very similar in appearance to those given by the reducing sugars. A second type of interference is due to substances that have mobilities close to those of the sugars, and which may affect R_F values by 'salting out'. This effect is only noticeable where the interfering substance is present in high concentration.

Interference of the third type, that due to electrolytes, was studied by Westall (1948), who showed that inorganic salts were to a large extent hydrolysed, and were separated as free acid and alkali particularly in chromatograms irrigated with phenol in the presence of ammonia. This resulted in the appearance of misleading spots on chromatograms sprayed with ammoniacal silver nitrate, since the free alkali caused local damage to the cellulose of the filter-paper when the chromatogram was heated to remove the solvent. False spots are also given by inorganic *anions*, particularly iodide and bromide. Where these are present in the test solution they may appear as hydrobromic acid and hydriodic acid, both of which react with ammoniacal silver nitrate to give rather diffuse spots of a violet-brown colour.

The separation of the ions of sodium chloride on paper chromatograms was an unexpected result, but the explanation may be quite simple. It is probable that the sodium ion has a high mobility when phenol is the mobile phase because it is transported as sodium phenate, which is very soluble in wet phenol. The mobility of the chloride ion appears to be depressed by the presence of ammonia, and this may be due to the formation of ammonium chloride, which would tend to remain in the aqueous phase. This explanation is rendered more plausible by the fact that, with collidine as the mobile phase, the mobility of the sodium ion is less than that of the chloride ion.

The separation of salts into cations and anions on paper chromatograms was also observed, independently, by Consden & Gordon (1948), who showed that the effect produced false yellow spots on chromatograms sprayed with ninhydrin solution for the detection of amino-acids. As a result of these interfering effects it is necessary, where inorganic salts are present in the test solution, to remove all electrolytes before attempting the chromatographic separation of the sugars. This may be carried out quite simply by passing the solution, first through a column packed with a suitable cation-exchanger (in its hydrogen form), in order

to remove the cations, and then through an anion-exchange column to remove the resulting free acids (Partridge, 1948*a*). It should be pointed out, however, that this procedure results in the removal of the amino-sugars and also any uronic acids that may be present.

The use of ion-exchange columns is necessary in almost all cases where unfractionated biological extracts are being examined, but in the analyses of the hydrolysis products of a polysaccharide, all that is necessary is to remove the free acid used for hydrolysis. The identification of sugars may be carried out in the presence of quite large concentrations of amino-acids without interference from this source.

SUBSTANCES RELATED TO THE SUGARS

The identification of various substances related to the sugars may from time to time be of value in carbohydrate studies. Many of these substances have similar mobilities to those of the sugars, and thus they may be identified by use of similar solvents and solvent mixtures. They may also appear on chromatograms carried out for the purpose of sugar analysis. As an example of this, a spot was found on the chromatogram of an extract of foetal blood of sheep that was afterwards identified as inositol (Partridge, 1948*a*). This substance gives a brown colour with ammoniacal silver nitrate, but the reaction is rather weak. Ascorbic acid, isoascorbic acid, reductone and hydroxytetronic acid all reduce ammoniacal silver nitrate at room temperature, and spots due to them may be revealed by this means. The indophenol dye commonly used to estimate ascorbic acid may also be used as a spraying reagent for the identification of these compounds. Dehydroascorbic acid reduces ammoniacal silver nitrate at 60–80° C. and forms a discrete spot on chromatograms when these are carried out in weakly acidic solvents.

SOLVENTS

Recently Jermyn & Isherwood (1949) studied a wide range of solvent mixtures for their efficiency in separating pairs of sugars, and obtained a marked improvement in the separations by irrigating paper chromatograms with ethyl acetate-acetic acid-water and ethyl acetate-pyridine-water mixtures. I am indebted to Drs Jermyn and Isherwood for the reproduction shown in Fig. 2, which is the reflex photograph of a very elegant chromatogram carried out in one of the solvent mixtures they recommend. This was ethyl acetate-pyridine-water mixed in the volume ratio 2 : 1 : 2, the solvent layer being used as the mobile phase of the chromatogram. The sugars have relatively low R_F values in this solvent mixture, and in order to achieve the maximum separation the paper strip was cut to a point at the bottom and the solvent allowed to drip

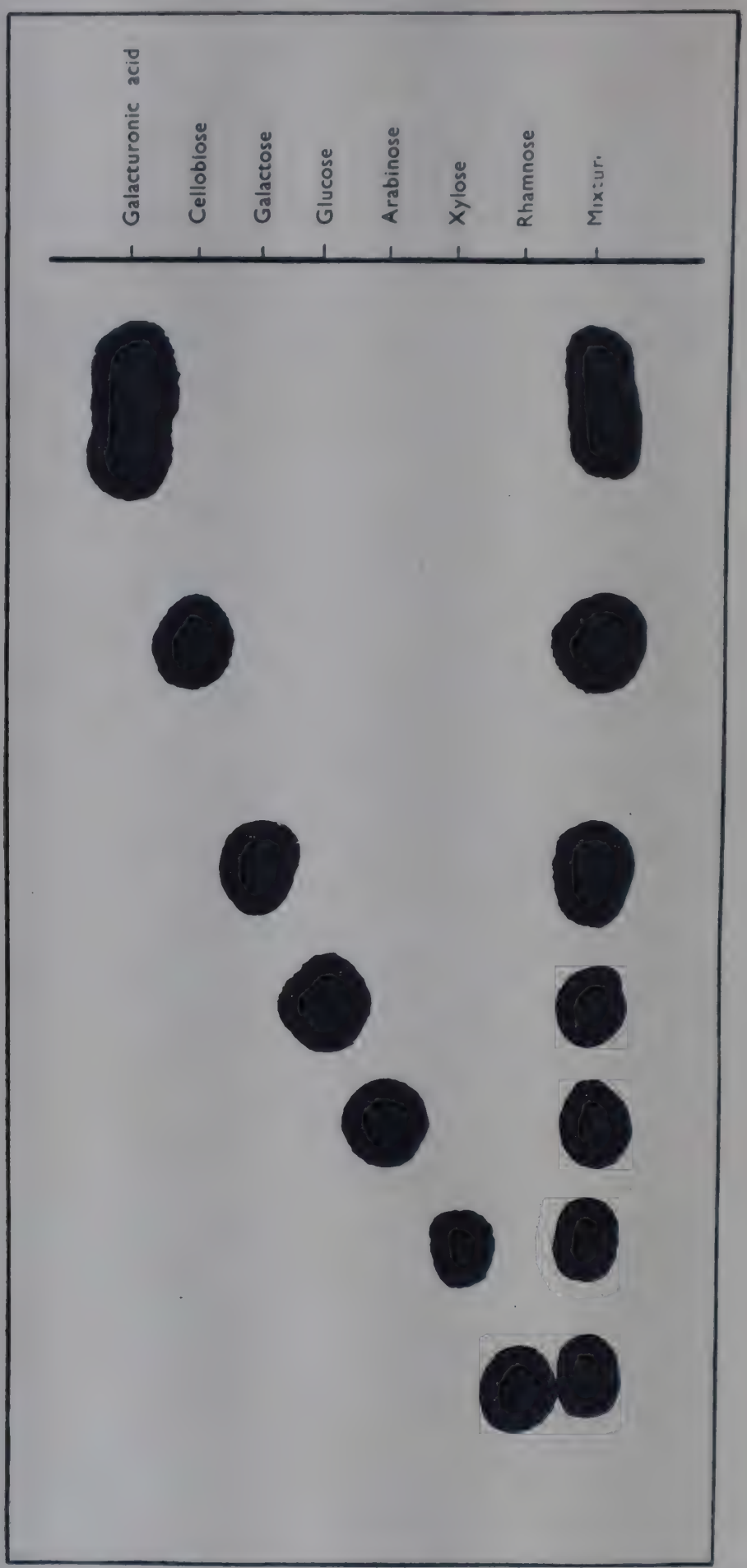


Fig. 2. Analysis of a mixture of seven sugars using ethyl acetate-pyridine-water mixed in volume ratio 2:1:2. The chromatogram was irrigated for 20 hr. at 20° C.

off the point until the fastest sugar reached a position near the bottom of the strip. The viscosity of the solvent mixture is low, and irrigation for only 20 hr. was sufficient to complete the chromatogram shown, which was carried out on a strip of Whatman no. 1 filter-paper 45 cm. in length.

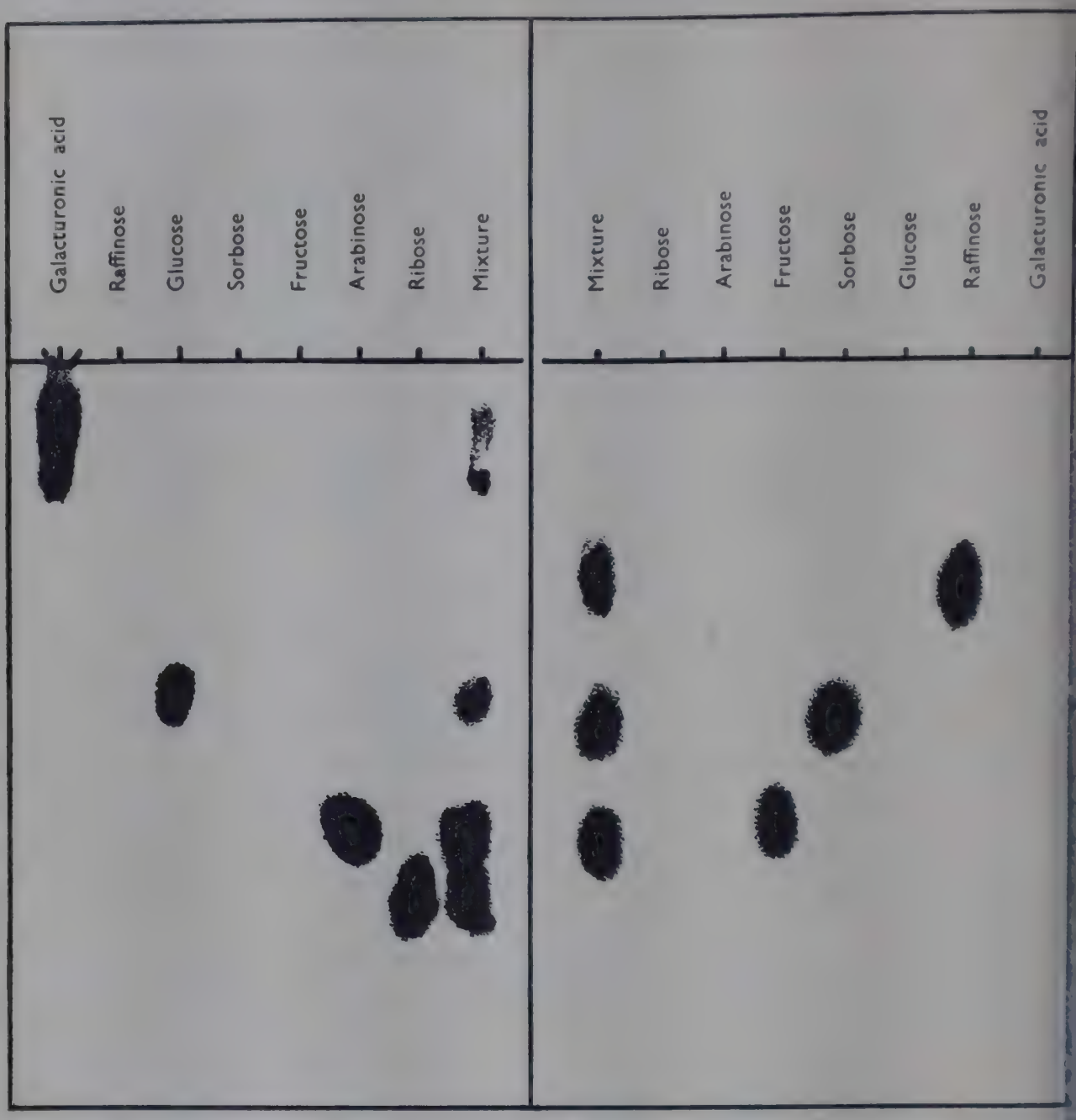
RECENT IMPROVEMENTS IN SPRAYING REAGENTS

The selectivity of the filter-paper method was improved by Forsyth (1948), who examined the colours produced by the sugars on heating with various phenolic reagents in the presence of hydrochloric acid. The use of strong mineral acids, however, has the disadvantage that the cellulose of the filter-paper is attacked, and the chromatograms require careful handling after they have been heated with the reagent. Chargaff, Levine & Green (1948) avoid this difficulty by spraying the chromatograms with *m*-phenylene-diamine dihydrochloride in 76 % alcohol. After heat treatment for 5 min. at 105° C. the sugar spots show a well-defined fluorescence in ultra-violet light, and as little as 10 μg. of the solution may be detected by this means.

Recently, in this laboratory, aniline hydrogen oxalate has been found to be a useful spraying reagent for the sugars. The reagent gives a very characteristic red colour with the pentoses and is also useful for the detection of aldohexoses, methylpentoses and uronic acids. Under controlled conditions, no colour is given with the ketoses, but the amino-sugars give a weak reaction. Fig. 3 shows a pair of duplicate chromatograms which were both prepared by irrigation with phenol. The chromatogram on the left-hand side was sprayed with aniline hydrogen oxalate, while that on the right was sprayed with naphthoresorcinol-trichloroacetic acid mixture. The chromatograms show the analysis of a mixture of seven sugars, three of which (fructose, sorbose and raffinose) contain the ketose structure. The naphthoresorcinol reagent revealed the ketose spots, while the remainder of the sugars were revealed by aniline oxalate. Thus, by the use of these two reagents, complete identification of this rather complicated mixture of sugars was possible without resorting to the preparation of further chromatograms using other solvent mixtures. It will be observed that sorbose and glucose, which have practically the same R_F value in phenol, may be differentiated by this means. This is rather important, since the R_F values of these two sugars lie very close together with most of the solvent mixtures commonly used for developing chromatograms.

The aniline hydrogen oxalate reagent is made up by dissolving 0·93 g. aniline in ethanol (50 ml.) and mixing the solution with an equal volume of 0·2 M-aqueous oxalic acid. After spraying, the chromatograms

are heated for 10 min. at 110° C. The colours produced are stable for a few weeks, but if a permanent record is required, photographic copies should be made by use of reflex paper. The reagent is very sensitive for the pentoses and aldohexoses and will detect as little as 1–2 μg. of either.



Solvent: phenol.
Spraying reagent: aniline hydrogen oxalate.

Solvent: phenol.
Spraying reagent: naphthoresorcinol-trichloroacetic acid.

Fig. 3. Two chromatograms illustrating the use of selective spraying reagents to assist in the differentiation of complex sugar mixtures.

The two spraying reagents, aniline hydrogen oxalate and naphthoresorcinol-trichloroacetic acid mixture, serve to detect most of the simple sugars, and since their action depends upon the presence of a furfural or substituted furfural structure in the molecule the reagents are more specific than ammoniacal silver nitrate. This is of considerable advan-

tage, since the reagents do not react with polyphenols or with the reducing impurities often found in solvents such as phenol or collidine and thus a very objectionable source of interference is eliminated.

The naphthoresorcinol-trichloroacetic acid reagent also serves to detect free hydroxymethylfurfural which may be formed as a result of severe acid hydrolysis of polysaccharides containing fructose. The substituted furfural forms a well-defined spot of high mobility (R_F, *c.* 0·85 in phenol) and gives a bright red coloration with the naphthoresorcinol reagent. Furfural itself, and methylfurfural, appear to be too volatile for successful separation on filter-paper.

APPLICATION TO QUANTITATIVE ANALYSIS

Quantitative applications of the method were explored by Flood, Hirst & Jones (1947), who eluted the spots given by sugars on filter-paper chromatograms and used micro-methods for estimating the sugar content of the resulting solution. In this way it was possible to analyse a mixture containing several sugars with an accuracy of $\pm 5\%$, using less than 1 mg. of material. Somogyi's micro-copper reagent was used for estimating the separated sugars, and since this method requires a rather large amount of reducing sugar the analysis was carried out on several spots of the mixture, which were placed side by side on the starting line of the chromatogram. A reference strip, which was detached and sprayed with ammoniacal silver nitrate, was used to locate the position of the components of the mixture and, with the aid of this, the main part of the chromatogram was cut so as to isolate the zones occupied by each component. The small strips of paper bearing the sugars were then each extracted with hot water in a refluxing apparatus and the analyses were carried out on the resulting solutions.

A similar method was developed by Hawthorne (1947). In this, oxidation with alkaline iodine at controlled pH was used to determine the reducing sugars. This method has some advantage, since the oxidation proceeds stoichiometrically with most aldoses, and can be carried out with as little as 40 μg. of sugar. Hawthorne extracted the strips bearing the sugars by irrigating them with water in a closed vessel in much the same manner as that used in the preparation of the chromatograms. In this way a strong solution of the sugars was obtained, and hence the need for subsequent concentration was avoided.

A very simple method for the quantitative analysis of the sugars was suggested by Fisher, Parsons & Morrison (1948), who showed that, under controlled conditions, the area of the spot due to a component varied directly with the logarithm of the amount of component applied. The spot areas may be measured conveniently by means of a planimeter from a reflex photograph of the chromatogram.

METHYLATED SUGARS

One of the most important applications of partition chromatography to the study of carbohydrates lies in the identification and estimation of the components of the mixture of methylated sugars resulting from 'exhaustive methylation' and hydrolysis of polysaccharides, and at an early stage D. J. Bell (1944) carried out the quantitative separation of tetramethyl, trimethyl and dimethyl glucose using columns of silica gel with chloroform or chloroform-butanol mixture as the solvent. Recently, Brown, Hirst, Hough, Jones & Wadman (1948) applied the filter-paper method to the identification of mixtures of methylated sugars, and examined a large number of such derivatives using ammoniacal silver nitrate to reveal the positions of the spots. The filter-paper method may also be applied to the quantitative analysis of the methylated sugars, but it was clear that a method capable of separating the sugars and their methylated derivatives in larger amounts would be desirable. This was required both for accurate quantitative work and for the purpose of separating sufficient material in a pure state for final identification by the determination of physical constants and the formation of characteristic derivatives. Such a method has recently been developed by Hough, Jones & Wadman (1948), who separated sugars and their methylated derivatives by partition chromatography on columns packed with finely powdered cellulose. The source of the cellulose was 'Whatman Ashless Filter Tablets', which were rubbed through an 80-mesh sieve and packed tightly into the filtration tube as a dry powder. The mobile phase was *n*-butanol saturated with water containing 1 % of ammonia. The solvent was allowed to percolate through the filtration tube until all the bands appeared in the effluent solution after traversing the entire length of the column. During this time fractions of the effluent solution were taken by changing the receiver at 30 min. intervals, and the flowing chromatogram thus obtained was analysed by paper chromatography, a small drop of the solution being taken from each of the receivers for setting up a unidimensional paper chromatogram. The remainder of the solution, after suitable collection of the fractions, was available for the isolation of chromatographically pure samples of the sugars, and for the quantitative estimation of the pure components by the usual analytical procedures. Used in this way, a column 12 in. in length served to separate three or four component mixtures of the sugars, with individual recoveries of 95–100 %.

APPLICATIONS

Examples of the practical application of the methods so far reported show that they may be applied successfully to the analysis of biological extracts of various kinds, and to the qualitative and quantitative analysis of the hydrolysis products of natural and methylated polysaccharides.

Qualitative analyses have already been carried out on the sugars occurring in fruit juices, foetal and maternal blood, seeds, the white of eggs, and mammalian muscle, and no doubt we shall learn of many other applications to different systems in the near future. In the field of the polysaccharides, qualitative and in some cases quantitative analyses have been carried out on the hydrolysis products of 'blood-group A substance', the specific polysaccharide of the 'Shiga' dysentery organism, the cell-wall polysaccharides of fruit, linseed mucilage and Chola gum. Investigations have also been made on various methylated polysaccharides, including methylated cherry gum, slippery elm mucilage and a sugar-beet araban. From the results already obtained it is apparent that the various methods of partition chromatography will be of wide application to investigations in the carbohydrate group and, with the refinements in technique which are now being introduced by many groups of workers, we may expect partition methods to prove themselves in carbohydrate studies and to gain the same acceptance as in the study of amino-acids and proteins.

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6. ANTHOCYANINS, FLAVONES AND OTHER PHENOLIC COMPOUNDS

By E. C. BATE-SMITH

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The possibility of applying filter-paper chromatography to the study of sap-soluble plant pigments was briefly discussed in an earlier paper (Bate-Smith, 1948). It was pointed out that the anthocyanidins and their mono- and diglucosides form spots well differentiated in their R_F values, and giving characteristic colour reactions with ammonia vapour. A flavone, presumably apigenin, and a yellow pigment, presumably butein, could be demonstrated together with cyanin and pelargonin in extracts of *Dahlia* petals. In addition, numerous unidentified components were visible, especially when viewed in ultra-violet light.*

In order eventually to be able to identify the pigments and related substances present in plant extracts, an extensive study has been made of anthocyanins, flavones and related polyphenolic compounds which, for the sake of brevity, will be referred to as 'C_{15} compounds'. These have been run under standard conditions, especially with respect to temperature, composition of the flowing solvent, and of the solvent in which the substance is applied to the paper. The chloride-ion content is of especial importance in its effect upon the R_F value of the anthocyanins. In the case of the aglucones of the anthocyanins (the anthocyanidins) it has been found that mineral acid in considerable concentration is needed to prevent decomposition during the run.

Most of the data have been obtained with Whatman no. 1 paper using butanol-acetic acid-water as the flowing solvent. The components are mixed in the proportion 40 : 10 : 50 by volume, and the supernatant layer is used after equilibration at the temperature at which the chromatogram is to be run. Equilibria in such ternary mixtures are rather sensitive to temperature, and in mixtures containing alcohols and acids esterification may occur, thereby adding an ester to the system and subtracting alcohol and acid from it. For accurate work it has been found necessary to take the following precautions:

(i) The mixture is allowed to stand for at least 3 days at the temperature at which it is to be used.

* The ultra-violet source employed in this work has been a G.E.C. 'Osira' lamp giving some emission in the visible violet.

(ii) A control substance is run with every chromatogram, and the solvent discarded as soon as this begins to show abnormal values.

(iii) The paper, before irrigation with the solvent, is equilibrated for 24 hr. with the vapour of the aqueous phase of the solvent-water mixture (Jermyn & Isherwood, 1949).

(iv) Chromatography is carried out at a temperature which is constant to within $\pm 0{\cdot}5°$, and for a constant time.

(v) Papers on which for any reason* the R_F value of the control substance differs by more than $\pm 0{\cdot}02$ from the standard value are disregarded. (It is not, as a rule, possible to correct the R_F value of an unknown substance for departure from the standard R_F value of the control, since different substances are affected to different extents by changes in conditions.)

When these precautions are taken, the R_F values of the anthocyanins and flavones and related substances in butanol-acetic acid show a remarkably regular relationship with chemical constitution. These substances can be considered as variously substituted derivatives of a basic flavan, or in a very few instances *iso*flavan, skeleton (Fig. 1):

With stepwise oxidation of the pyran ring we have:

Flavanols, e.g. catechin.

Flavanones, e.g. butin.

Chalcones, e.g. butein. (N.B. Chalcones are open-ring isomers of the flavanones.)

Flavones, e.g. luteolin.

Flavonols, e.g. quercetin.

Flavylium salts (anthocyans), e.g. cyanidin chloride.

In addition, specimens of the benzalcoumaranones, leptosin and leptosidin (Geissmann & Heaton, 1943, 1944) have been available for examination. These also are C_{15} compounds. Altogether more than fifty C_{15} compounds have been studied.

The naturally occurring representatives of these classes, with the single exception of flavone, are hydroxylated. In many of them one or more of the hydroxyl groups is methylated, and in most of them one or more of the hydroxyl groups is attached to a sugar residue by a glycosidic linkage. As a further complication, the sugar residue, in the anthocyanins at least, is frequently esterified with an aromatic or aliphatic acid, in which case it is referred to as 'acylated'.

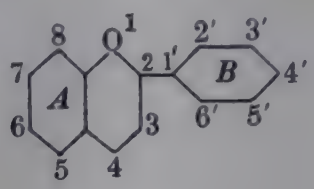
The R_F values show the following regularities:

(1) C_{15} compounds of the classes enumerated above having the same number of hydroxyl groups have approximately the same R_F value, the R_F value falling with each additional hydroxyl group.

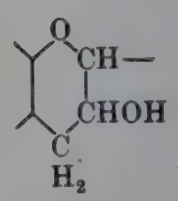
* The R_F value also varies from batch to batch of filter-paper, but is reasonably constant within a batch as usually purchased by the ream.

(2) The glycosidic combination of sugars other than rhamnose causes approximately the same fall in R_F value as the addition of a hydroxyl group. This effect is additive, but the formation of a bioside causes a lesser fall in R_F value than that of a diglycoside.

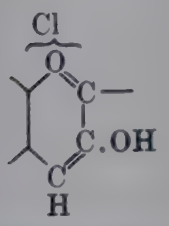
(3) The glycosidic combination of rhamnose causes either a slight rise or a slight fall in R_F value.



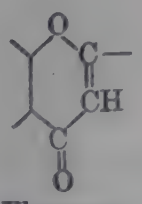
'C_{15}' skeleton



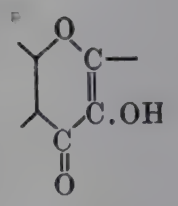
Catechins
(Flavanols)



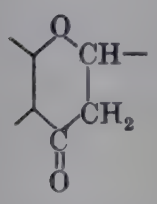
Anthocyanidins
(Flavylium chlorides)



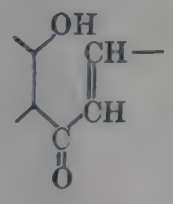
Flavones



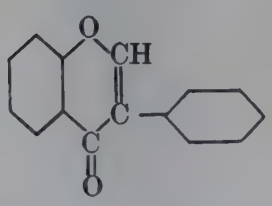
Flavonols



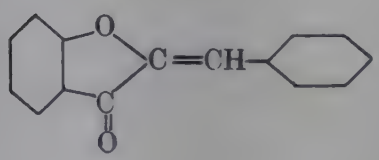
Flavanones



Chalcones



Isoflavones



Benzalcoumaranones

Fig. 1. Types of C_{15} compounds studied.

(4) Methylation of a hydroxyl group causes a rise in R_F value varying as a rule between one-third and two-thirds of the rise which would result from the loss of the hydroxyl group.

(5) The effects of acylation can only at present be inferred from the occurrence of numerous anthocyanin spots on the chromatograms of natural extracts which have the colour character of known anthocyanins, and yield known anthocyanins on mild hydrolysis, but differ

in R_F value. The latter may be either greater or less than the R_F value of the unsubstituted anthocyanin, according to the nature of the acylating group. Thus aromatic acids (e.g. *p*-hydroxybenzoic, *p*-hydroxycinnamic, sinapic acids) would be expected to cause a rise in R_F value, aliphatic acids (e.g. malic, glucuronic acids) a fall.

In addition to the instances of *Peristrophe speciosa* and *Tibouchina semidecandra* quoted previously (Bate-Smith, 1948), multiplication of anthocyanin types by presumed acylation has been met with in many cultivated plants, conspicuously in *Brassica oleracea* var. *rubra*, *Pelargonium zonale* Hort. varieties, and *Dahlia* spp.; and even when single anthocyanins are present they frequently differ in properties from the standard. It will be seen below that polyphenols and polyphenolic acids occur in the free state and perhaps also as glucosides in petal extracts, in which they can be demonstrated by characteristic reactions when chromatographed on paper.

CHROMATOGRAPHY OF SIMPLER PHENOLIC SUBSTANCES

The close correlation between structure and R_F value in butanol-acetic acid in the cases described above suggested that an examination of phenolic compounds derived from benzene might yield results of value in analytical chemistry.

Since these substances are colourless, to ascertain their position they needed to be developed on the paper. This could normally be done with the aid of ammoniacal silver nitrate. The earlier forecast (Bate-Smith, 1948) that aromatic compounds having two or more hydroxylic substituent groups in the nucleus could be developed on the paper in this way has been borne out. Those in which the two hydroxyls are *ortho* or *para* with respect to one another reduce in the cold, otherwise reduction takes place on heating, as in the chromatography of sugars by Partridge's method. This general rule is obeyed by all the phenolic substances examined, except syringic acid, which reduces strongly in the cold, although two of its three hydroxyls are methylated, vanillic acid which reduces slightly in the cold, and *p*-hydroxybenzoic acid which, although only monohydroxylic, reduces on heating.

The phenolic compounds also give colours with ferric chloride; in some instances, e.g. protocatechuic acid, the colour is so characteristic as to be itself of diagnostic value. The position of cinnamic acid, which does not reduce silver, was detected by its yellow colour reaction with ferric chloride; that of benzoic acid by spraying with bromcresol green. (Iridin, irigenin, tectoridin and tectorigenin are also conveniently detected with ferric chloride.)

A property of phloroglucinol and its carboxylic acid derivative which is likely to be of great diagnostic value is its distinctive blue fluorescence under the ultra-violet lamp which is visible only in presence of ammonia. *o*-Coumaric acid has an exceedingly brilliant greenish fluorescence both in presence and absence of ammonia.

From the systematic point of view, the data obtained with polyhydroxybenzenes, their carboxylic acids and methyl esters, support those drawn from the R_F values of the anthocyanins and flavones. The R_F values of substances differing only in the number of hydroxyl groups depend primarily on the number and only secondarily on the position of such groups.

Methylation of hydroxyl groups causes an increase in R_F rather less than would be caused by complete removal of the groups. A carboxylic group causes a decrease in R_F much less than would be caused by an additional hydroxyl group. It is to be anticipated that glucosidation of hydroxyl groups would follow the same rules as in the case of the more complex polyphenols discussed earlier.

BEHAVIOUR IN *m*-CRESOL-ACETIC ACID AND OTHER SOLVENTS

The chromatographic behaviour of the C_6 and C_{15} compounds has been tested with most of the solvents now in common use: phenol, pyridine, collidine, ethyl acetate, various alcohols, etc., alone or with the addition of acid or ammonia. Only one other solvent has been found to give satisfactory definition of spots and absence of trailing with the C_{15} compounds, viz. *m*-cresol-acetic acid-water (50 : 2 : 48) equilibrated as for the butanol-acetic acid mixture; with this solvent there is a regularity of behaviour with chemical constitution, but the spots are more widely spaced and, fortunately, the R_F values of substances containing the same number of hydroxyl groups fall in a different order from that in butanol-acetic acid. This should facilitate the identification of unknown substances by two-dimensional chromatography; but it should be noted that the C_{15} compounds tend to form elliptical spots which, when run two-dimensionally, would cover a large area.

The R_F values at 20° C. in these two solvents of all the substances which have been examined to date are set out in Tables 1 and 2.

CHROMATOGRAPHY OF NATURAL EXTRACTS

It has been borne in mind that if this method is to have extended practical use in plant physiology, elaborate equipment and delicate control of temperature cannot be envisaged. However, as has been pointed out earlier, carefully controlled conditions are required for the

accurate determination of R_F values, and when these conditions cannot be satisfied the measurement of R_F values can be used as a rough guide only; for unambiguous identification reliance must be placed on colour reactions, and especially on the acquisition of specimens for reference.

Table 1

Substances	R_F values at 20° C. Butanol-acetic acid	R_F values at 20° C. *m*-Cresol-acetic acid
Flavones and flavonols:		
Apigenin	0·92	—
Apiin	0·66	0·80
Chrysin	0·97	(1·0)
Diosmetin	0·89	—
Diosmin	0·55	—
Fisetin	0·72	0·40
Galangin	0·92	0·92
Gardenin	0·82	0·96
Iridin	0·73	0·90
Irigenin	0·96	0·95
Isoquercitrin	0·68	0·47
Kaempferitrin	0·75	0·72
Kaempferol	0·90	0·61
Luteolin	0·88	0·68
Morin	0·87	0·62
Myricetin	0·43	0·05
Myricitrin	0·72	0·24
Nor-gardenin	0·10	0·0
Quercetagetin	0·40	0·06
Quercetin	0·74	0·27
Quercimeritrin*	0·59	0·42
Quercitrin	0·80	0·53
Rhamnazin	0·80	0·97
Rhamnetin	0·77	0·78
Rutin	0·58	0·26
Tambuletin	0·45	0·83
Tectoridin	0·68	0·87
Tectorigenin	0·94	0·98
Miscellaneous C_{15} compounds:		
Butein	0·85	0·62
D-Catechin	0·74	0·22
Cyanomaclurin	0·88	—
L-Gallocatechin	0·47	0·0
Leptosidin	0·79	0·83
Leptosin	0·46	—
Phloretin	0·96	0·80
Phloridzin	0·80	0·58
Benzene derivatives:		
Benzoic acid	0·92	0·93
Catechol	0·91	0·74
Cinnamic acid	0·94	0·92
o-Coumaric acid	0·94	0·82
Gallic acid	0·68	0·08
Hydroquinone, *see* Quinol	—	—
m-Hydroxybenzoic acid	0·91	0·72
p-Hydroxybenzoic acid	0·90	0·72
Orcinol	0·91	0·75
Phloroglucinol	0·76	0·16
Phloroglucinol carboxylic acid	0·55	0·06
Protocatechuic acid	0·85	0·35
Pyrocatechol, *see* Catechol	—	—
Pyrogallol	0·77	0·38
Quinol	0·88	0·69
Resorcinol	0·91	0·63
β-Resorcylic acid	0·93	0·54
Salicylic acid	0·95	0·84
Vanillic acid	0·92	0·81

* Two specimens of this flavone have been obtained both showing two components. The values quoted relate to the component in the two samples showing the best agreement.

It is not essential to obtain the reference substance in a pure condition; it is sometimes sufficient to compare the chromatogram of an unknown mixture with that of an extract containing pigments of known constitution. (The values quoted for diosmin, diosmetin, tectoridin and tectorigenin in this paper were obtained with extracts of dried hyssop and the roots of *Iris tectoris*, respectively. Very little purification of the extracts was necessary.)

Most of the C_{15} compounds occur in nature as glycosides, and many of the anthocyanins are acylated. With such possibilities of variation it is difficult to envisage being able to identify by direct chromatography each and every glycosidic and acylated variant of the basic C_{15} com-

pound, but, since mild hydrolysis is sufficient as a rule to split off these groups, it should usually be possible to identify the parent substances.

The method of extraction with 1% Aq. HCl used by Robinson & Robinson (1931) is an admirable one for the present purpose, since mild hydrolysis (100° C. for 15 min.) can be carried out immediately on the

Table 2. *Anthocyanins and anthocyanidin chlorides*

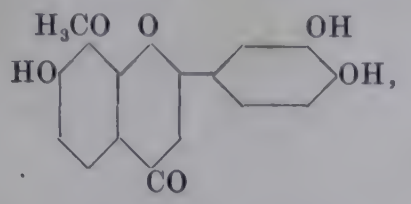
		R_F values in		
		Butanol-acetic acid	2N-HCl	*m*-Cresol-acetic acid
Apigeninidin		0·82	—	1·0
Cyanidin:		—	0·69	—
-3-glucoside	Chrysanthemin	0·33	0·27	Trail
-3-rhamnoglucoside	Antirrhinin	0·37	0·28	0·25
-3-gentiobioside	Mekocyanin	0·29	0·22	0·18
-3, 5-diglucoside	Cyanin	0·16	0·08	0·19
Delphinidin:		—	0·35	—
-3-monoside	ex *Verbena*	0·16	0·14	0·11
-3, 5-diglucoside	Delphin	0·11	0·06	0·03
Hirsutidin:		—	0·72	—
-3-glucoside		0·61	—	—
-3, 5-diglucoside	Hirsutin	0·38	0·07	0·69
Malvidin:		—	0·53	—
-3-glucoside	Oenin	0·40	0·23	0·75
-3-galactoside	Primulin	0·40	0·24	0·76
-3, 5-diglucoside	Malvin	0·22	0·07	0·54
Pelargonidin:		—	0·80	—
-3-glucoside	Callistephin	0·59	0·52	0·67
-3, 5-diglucoside	Pelargonin	0·34	0·20	0·42
Peonidin:		—	0·72	—
-3-glucoside	Oxycoccicyanin	0·47	0·31	0·72
-3, 5-diglucoside	Peonin	0·26	0·10	0·48

extract. It has the disadvantage of introducing mineral acid. This affects the R_F of substances, especially the anthocyanins (cf. p. 62), which move behind the acid front. (In the case of HCl in butanol-acetic acid the HCl front has approximately $R_F = 0{\cdot}4$.) A useful alternative solvent for extraction is the aqueous phase of the butanol-acetic acid-water mixture, but if hydrolysis is to be employed in any case, there is no advantage in avoiding the HCl in the extract.

The complexity of the chromatograms usually produced by petal extracts is illustrated in Pl. 1, fig. 1.

In the case of *Coreopsis* species, as a result of the recent work of Geissmann (1941, 1942) and Geissmann & Heaton (1943, 1944), we have, perhaps, the best opportunity of identifying the substances responsible for the spots on the chromatogram. From *Coreopsis Douglasii* Hall (= *Leptosyne Douglasii* DC.), Geissmann isolated butein; from *Coreopsis gigantea* Hall (= *Leptosyne gigantea* Kell.), butein and a second polyhydroxychalcone; and from *Cosmos sulphureus* Hort., 'Orange Flare' (a butein glucoside), luteolin and a quercetin glycoside,

probably isoquercitrin. From *Coreopsis grandiflora* Nutt., Geissmann & Heaton isolated the coumaranone leptosin, its aglucone leptosidin, luteolin and a flavanone of the probable structure



i.e. 8-methoxybutin. Through the courtesy of Prof. Geissmann I have been able to obtain specimens of the new pigments and have studied their chromatographic behaviour.

If we examine the chromatogram of an extract of *Coreopsis grandiflora* petals in the light of this information as to the substances likely to be represented in it, we can immediately identify leptosin and leptosidin, but the flavanone does not appear to be present. Instead, we have a spot corresponding with butein, and another corresponding very nearly with spots in *Leptosyne Douglassii*, *Cosmos sulphureus* and *Dahlia* chromatograms which must be assigned to butein glucoside. It seems probable, therefore, either that, as Geissmann suggests, the new flavanone occurs in the petals as a chalcone 3′-methoxybutein (N.B. 3′ position in chalcone enumeration ≡ 8 position in flavan enumeration), which would behave very much like butein, or that the pigment in the specimens I have studied is butein itself. The balance of evidence favours the latter, and I shall refer to this component as butein. There are still a number of spots remaining to be identified, most of them in the range of lower R_F values. A clue to the nature of these is provided by hydrolysis of the extract, and by serial examination of the petals of the developing flower. The latter provides the more conclusive evidence, reproduced in Pl. 1, fig. 2.

In this chromatogram the paper has been sprayed with ammoniacal silver nitrate and heated. Each separate chromatogram represents the pigment constitution of a single petal from one and the same flower, which remained on the plant while successive petals were removed. The first, starting from the top, was the first petal to open, single petals then being taken on successive days. The weather being at the time cold and dull, the flower had not withered by the time the last petal was removed. The feature to which attention is drawn is the strength of the butein spot in the first petal (extreme right), gradually diminishing almost to zero in the tenth, while the butein glucoside spot (centre) increases in strength. Equally evident in the untreated chromatogram (but not easily reproduced for publication) is the increase in leptosin at the expense of leptosidin and the appearance of new spots in the lower range of R_F corresponding in colour reactions with butein and leptosin.

These can only be higher glucosides (diglucosides or biosides) of these substances. A great deal of the complexity of the chromatogram can therefore be accounted for in terms of the multiple glucosidation of only two primary constituents.

When, as is usually the case, anthocyanins are present along with these glycosidic anthoxanthins, the chromatogram may become very complicated in the range of low R_F. There are several ways in which the problem of characterization and identification can be simplified:

(1) The anthoxanthins can be removed from the HCl extract by exhaustive extraction with ethyl acetate, leaving the anthocyanins in the aqueous residue.

(2) The chromatogram can be run completely off the papers, until the anthocyanin spots extend over its full length.

(3) A second solvent (e.g. *m*-cresol-acetic acid) can be used to give a second chance of separating overlapping spots.

The anthocyanins seem, especially in cultivated plants, to occur in rather complex mixtures, and even when they occur singly, to differ in R_F value and other properties from the simple natural or synthetic substances. A case in point is the garden 'geranium' 'Kowalewski', which contains one predominant anthocyanin readily identified as a pelargonidin derivative, but agreeing neither with pelargonin nor callistephin (the 3-monoglucoside) in chromatographic behaviour. For a garden 'geranium' this variety has an exceptionally simple anthocyanin make-up. The species *Pelargonium inquinans* has three, the hybrid *P. Kewense* six, the variety 'Paul Crampel' five, both cyanin and pelargonin being represented in each case. *P. zonale*, the parent (probably with *P. inquinans*) of the garden 'geranium', has one anthocyanin, a modification of cyanin. In the absence, at present, of further information, it is considered that these modifications are most likely due to acylation. The free anthocyanidins have not been encountered in petal extracts. There is no evidence of gain or loss of glycosidic groups such as has been demonstrated in the *Coreopsis* anthoxanthins.

The chromatograms of many petal extracts, when treated with *strong* ammonia vapour and viewed under the ultra-violet lamp, show spots having the distinctive soft blue fluorescence of phloroglucinol already mentioned. One such spot, in the case of *Pelargonium* varieties, has the R_F value of phloroglucinol itself both in butanol- and *m*-cresol-acetic acid, and there seems no reason to doubt that phloroglucinol is actually present in these flowers. Another frequently occurring spot of this type has R_F 0·2–0·25, a value which suggests that it might be a glycoside of phloroglucinol.

A spot which strongly reduces ammoniacal silver nitrate in the cold, having R_F 0·3 approximately (butanol-acetic acid, 17° C.), has been

found in several varieties of garden 'geranium'. On hydrolysis this spot moves to the position of gallic acid, and its probable identity with gallic acid is shown by parallel chromatography with the pure substance Pl. 1, fig. 3. The original substance might well, from its position and ease of hydrolysis, be a glycoside of gallic acid.

A spot agreeing in R_F value and all other characters with kaempferol appears in hydrolysed extracts of *Pelargonium* species and varieties. Since it is absent from the unhydrolysed extract, it is presumably glycosidic in the natural state.

In addition to these demonstrations of the possibility of identifying unknown C_6 and C_{15} polyphenols in natural extracts, it has been possible in several instances to demonstrate on the filter-paper the presence, along with the expected products of hydrolysis, of breakdown products of the synthetic anthocyanins, and to obtain some information as to their probable nature. Thus, to quote only a few examples, in a hydrolysate of hirsutin a spot agreeing in its NH_3–ultra-violet colour and R_F value with phloroglucinol carboxylic acid (run simultaneously) was observed. In similar hydrolysates of peonin, malvin and hirsutin, spots of R_F 0·25, 0·26 and 0·32 respectively, also giving the phloroglucinol fluorescent reaction, could be detected. Cyanidin monoside subjected to alkali fission showed spots agreeing with phloroglucinol and protocatechuic acid. It seems likely that this technique will prove of value in the recognition of the split products of these and other complex substances of natural origin, and may serve as a help to their identification and as a guide to their chemical constitution.

I would like to acknowledge with gratitude the gift of specimens from Mrs R. Meares; Profs. W. Baker, T. A. Geissmann, T. R. Seshadri, A. R. Todd; Drs A. E. Bradfield, E. J. Cross and F. D. Tollenaar, and the assistance of Messrs D. F. Elsden and R. G. Westall in the experimental work.

I wish especially to acknowledge the stimulating advice given to me by Dr S. M. Partridge throughout the investigation.

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CONTRIBUTION TO DISCUSSION OF ABOVE PAPER

Separation of Porphyrins by Partition Chromatography. By C. RIMINGTON. *Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1*

The application of partition chromatography to the porphyrins by Miss R. E. H. Nicholas, working in my Department (Nicholas & Rimington, 1949), has brought out some interesting physico-chemical relationships. A large number of solvent systems was tried, but good results were only obtained by using (*a*) lutidine/water in an atmosphere of ammonia, or (*b*) lutidine/water alone, after application of the pigments as their ammonium or lutidine salts.

Under these conditions, good separation was achieved into groups according to the number of COOH groups in the molecule. At 19° the R_F values are, under condition (*a*):

	No. of COOH groups	R_F
Uroporphyrins	8	0·3
Coproporphyrins	4	0·6
Protoporphyrin, mesoporphyrin, haematoporphyrin, deuteroporphyrin, etc.	2	0·8
Phylloerythrin	1	0·9
All porphyrin esters	0	1·0

If the R_F values are plotted against the number of COOH groups, they are found to have a linear inverse relationship. The identical R_F values of the di-carboxyporphyrins indicate that, under these conditions, the nature of the side chain substituents has no effect in determining partition. This is further illustrated by the fact that the position isomers (I and III series) have identical R_F values. The sensitivity of the method is great; as little as 0·5 μg. of porphyrin may be recognized by observing the fluorescence of the spot under ultra-violet irradiation.

The copper co-ordination complexes have the same R_F values as their parent porphyrins, but the haems (Fe complexes) behave as if they contained one more COOH group. This is in accordance with expectation, since the work of Morrison & Williams (1941) has demonstrated the presence in the haems of a titratable additional charge attached to the iron atom. No such charge is present in the copper complexes.

Whilst the method has not yet been developed to achieve separation of the porphyrin isomers, it has yet afforded results of considerable value. Thus the presence has been demonstrated of a small quantity of uroporphyrin in normal human urine—and of an unknown porphyrin, apparently containing five COOH groups, in the urine of a case of acute porphyria.

The porphyrins produced by cultures of *C. diphtheriae* include coproporphyrin, some uroporphyrin and smaller quantities of two unknown porphyrins apparently containing five and six COOH groups respectively (cf. Gray & Holt, 1948).

On the other hand, examination of a specimen of H. Fischer's conchoporphyrin, kindly supplied by Prof. Waldenström, revealed only uroporphyrin together with some coproporphyrin and none of the penta-carboxyporphyrin reported by Fischer & Jordan (1930).

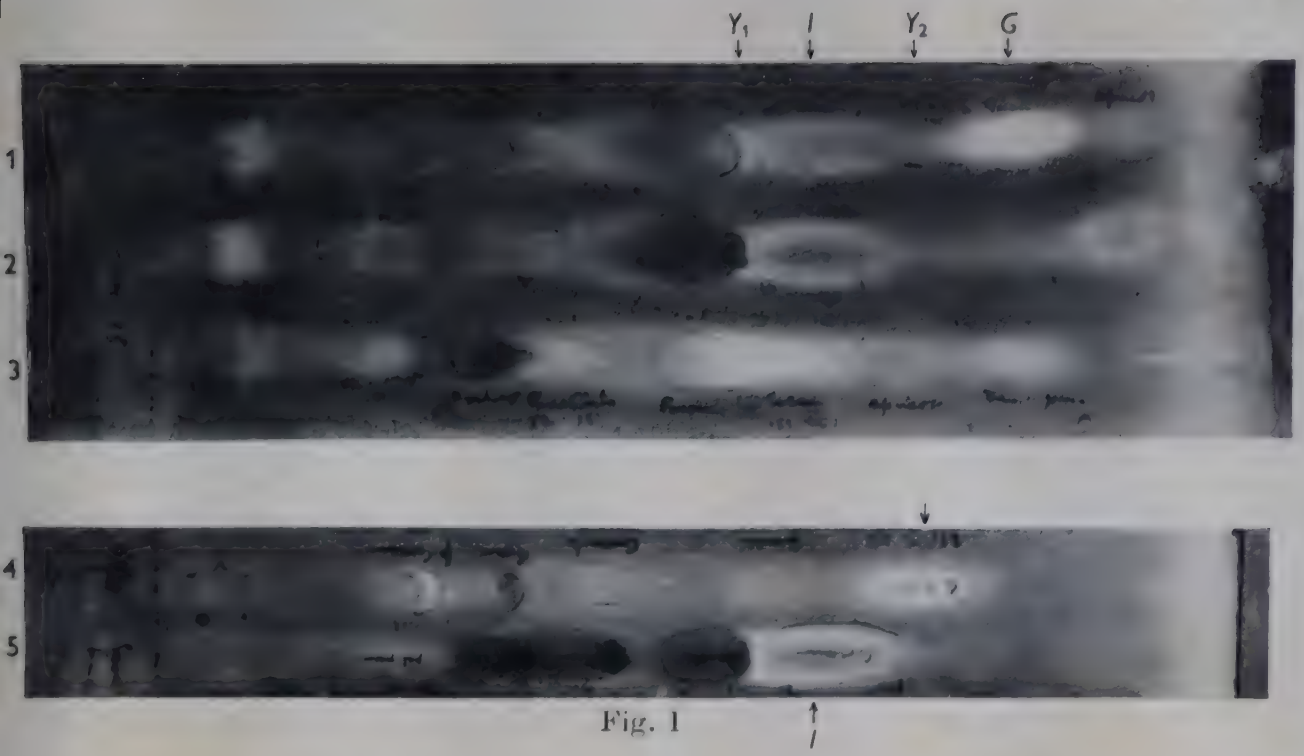

Fig. 1

Fig. 2

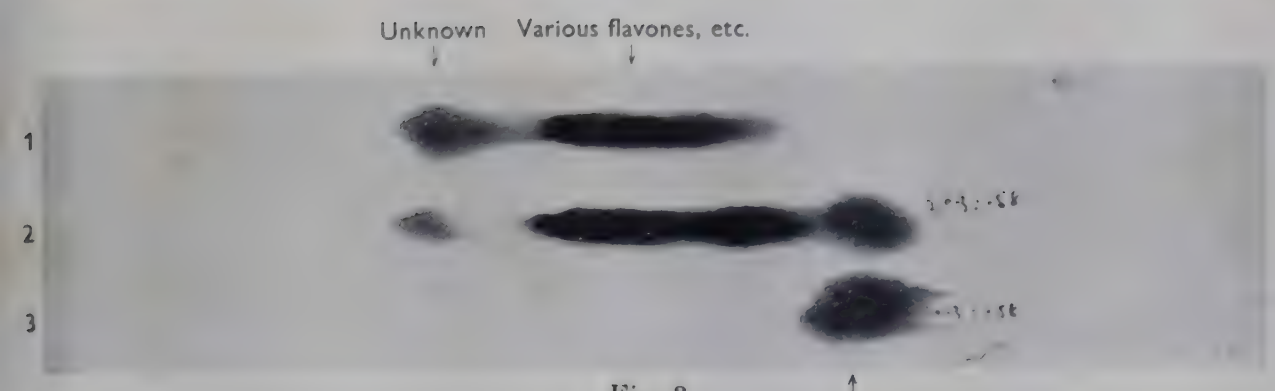

Fig. 3

Some uncertainty exists concerning the zoological identification of the shells investigated by Fischer; therefore a wide survey has been made with Dr Comfort of the occurrence of porphyrins in marine shells including *Pteria radiata* and *P. vulgaris*, but no porphyrins other than uroporphyrin and coproporphyrin have so far been encountered.

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EXPLANATION OF PLATE

Fig. 1. Chromatograms of extracts of pale yellow (1, 3) and white (2, 4, 5) *Dahlia* petals photographed in ultra-violet light. 2 = white sport of 1, showing loss of at least three constituents as a result of loss of single genetic factor *Y*. 3 and 4 = F_1 and F_2 offspring respectively of 1. Y_1 and Y_2 = yellow constituents, probably butein glucosides; *I* = 'ivory' constituent, probably apigenin glucoside, *G* = colourless constituent fluorescing bright green in ultra-violet light, probably a flavone.

Fig. 2. Chromatogram sprayed with Aq. NH_3-Ag.NO_3 and heated. 1 = glucose marker; 2–11 = extracts prepared from single petals of *Coreopsis grandiflora*. *G* = glucose, *F* = fructose, *L* = leptosin, *BG* = butein glucoside, *B* = butein.

Fig. 3. Chromatogram sprayed with Aq. NH_3-Ag.NO_3, in the cold. 1 = 1 % HCl extract of geranium 'Paul Crampel'; 2 = the same hydrolysed 30 min. at 100° C. 3 = gallic acid.

7. PARTITION CHROMATOGRAPHY OF ORGANIC ACIDS, PURINES AND PYRIMIDINES

By S. R. ELSDEN

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THE LOWER FATTY ACIDS

The lower fatty acids differ from one another in their partition coefficients, and it is to be expected therefore that they will separate on the partition chromatogram. But before discussing the various attempts to exploit this, I think it not out of place to discuss the relevant physicochemical properties of these compounds so that the difficulties which are encountered in this work can be assessed, and the methods which have been proposed evaluated.

The partition coefficients of the fatty acids between organic solvents and water are not constant but vary directly with the concentration of the acids; this is due to the readiness with which these compounds associate in organic solvents, and to the fact that, being weak acids, they are partially ionized in solution. From these facts it can be inferred that, when placed on the partition chromatogram, they will produce bands with well-defined leading edges and pronounced tails. Martin & Synge (1941) demonstrated that the R_F value is related to the partition coefficient, and it can be predicted that the R_F values of the fatty acids will vary with the concentration. These predictions have been confirmed in practice.

As the homologous series is ascended the partition coefficients increase rapidly. This at once sets a limit to the range of the simple chromatogram, and inspection of the partition coefficients of the lower fatty acids between a variety of organic solvents and water indicates that a separation of acids containing more than five carbon atoms is not to be expected, and that a resolution of mixtures of isomers is most unlikely, since the isomers of a given acid have similar partition coefficients. This is found to be the case experimentally, and it has proved impossible to achieve a separation of butyric acid from valeric acid save under exceptional circumstances where the concentration of the latter is greatly in excess of that of the former.

The pK's of the lower fatty acids are around 4·6, and if an indicator is to be used on the column it must, for maximum sensitivity, have a pK of the same order. Lester Smith (1942) recommended bromo-cresol

green, and this has been used by most analysts. However, it is not as sensitive as could be desired, particularly with butyric acid, as most of this acid is in the non-polar phase; it is also readily leached by CB 5 (chloroform containing 5 % (v/v) *n*-butanol). An indicator with a somewhat higher pK would, I think, be preferable, although Lester Smith in a memorandum has pointed out that such a procedure is likely to accentuate tailing. Very little work has been done on the problem of suitable indicators, for investigators have been too eager to obtain a tool which would serve their purpose rather than 'waste' time on such niceties.

Silica gel has been the main carrier used. It has the advantage that it is both cheap and easy to prepare and that fatty acids are not readily adsorbed on it. The gel, as prepared by the Leeds group, is acid to bromo-cresol green, and it is necessary to incorporate the appropriate amount of sodium hydroxide in the indicator solution to adjust the pH of the non-polar phase to 4·6; however, the incorporation of alkali has an adverse effect in that it increases the partition coefficient of the fatty acids in favour of the organic phase by a salting-out effect. Secondly, local patches of high alkalinity may arise, due to faulty mixing of gel and indicator, and cause incomplete recoveries. This can be overcome by running a fast-moving acid, e.g. *n*-valeric, through the column before use. It would be convenient were it possible to prepare gels of known reaction, but so far this has not been achieved; ageing in the appropriate buffer followed by washing with distilled water has no effect on the final pH of the gel.

The remaining property of the fatty acids which I wish to consider is their volatility. The fact that they are volatile complicates the extraction procedure, for they can only be concentrated as their salts; yet, when this is done, the problem of extraction still remains. The minimum volume of solvent found necessary to ensure quantitative extraction is about 25 ml. CB 5, far larger than can be conveniently accommodated on a 3–4 g. column. Aliquots have, therefore, to be taken for analysis, with the consequent introduction of further measurements and a reduction in the sensitivity of the method. For sharp separations a volume not greater than 3 ml. must be placed on a 3 g. column; larger volumes result in the bands becoming broad and diffuse with a considerable loss in resolution.

The initial purification of the acids is, in my experience, best achieved by the method of Friedemann (1938). This method has the added advantage that formic acid, which can only be developed off the column with high concentrations of butanol, is removed; this is no loss, as there are available reasonably specific chemical methods for the estimation of formic acid.

The simple silica gel chromatogram, with bromo-cresol green as the

indicator, will give reasonable results with mixtures containing acetic, propionic and butyric acids (Elsden, 1946). This method was based largely on that described by Lester Smith (1942) for qualitative work. There are three major criticisms which can be made of it: (1) The limited range—i.e. that acids above butyric cannot be separated; though it should be borne in mind that mixtures consisting of propionic and valeric acids would be well separated. (2) The relatively large amounts which are required for estimation due in part to the small volume of extract which can be placed upon the column, and in part to the volume of solvent required for quantitative extraction. (3) The fact that acetic acid has to be estimated by difference.

The problem of extending the range of the method has been studied by three groups of workers, and the procedures recommended all involve radical modifications of the immobile phase. Scarisbrick, Baldwin & Moyle (1948), and Moyle, Baldwin & Scarisbrick (1948) have shown that silica gel columns, heavily buffered with alkaline phosphate mixtures, can be used to separate all the acids up to C_8. Peterson & Johnson (1948) have shown that the fatty acids up to C_{10}, in the solvent system concentrated sulphuric acid-benzene, have partition coefficients such that the separation of these acids on a chromatogram is a practical proposition; in addition, they have introduced a new carrier, Celite 545, in place of silica gel. In both these methods, because of the nature of the non-polar phase, the valuable indicator technique has had to be abandoned and the columns 'run blind'. Ramsey & Patterson (1948) have developed a method for separating this group of acids while still retaining indicator technique, but, as I have been unable to obtain a copy of their communication, I do not think it wise to comment further on it.

The method employing a buffered polar phase requires three columns for the separation of all the acids up to octanoic:

Column I buffered with a 2:1 mixture of 2 M-K_2HPO_4:2 M-KH_2PO_4.

Column II buffered with a 2·5:3·5 mixture of 2 M-K_2HPO_4:2 M-K_3PO_4.

Column III buffered with a 2 M-K_3PO_4.

Columns of 5 g. of gel and 3 ml. buffer are used, made up in CB 1; three solvent mixtures are used for development of each column—CB 1, CB 10 and CB 30.* Column I separates acetic, propionic and butyric acids from the higher acids which pass out of the column in the first few ml. of effluent; CB 1 is used to elute butyric acid, CB 10 propionic acid and CB 30 acetic acid. Column II separates butyric, valeric and hexanoic acids from the higher acids which pass out of the

* CB 1 = chloroform containing 1/(v/v) *n*-butanol.
CB 10 = chloroform containing 10/(v/v) *n*-butanol.
CB 30 = chloroform containing 30/(v/v) *n*-butanol.

column undifferentiated in the first few ml. of effluent, whilst acetic and propionic acids are fixed permanently in the aqueous phase; CB 1 brings out hexanoic acid, CB 10 valeric acid and CB 30 butyric acid. Column III is used to separate heptanoic, hexanoic and valeric acids, octanoic being by-passed in the 'first runnings'.

These buffered columns have a number of advantages over the simple column; the bands are symmetrical and there is no tailing; the acids are determined directly in every case; relatively large volumes of extract—up to 20 ml.—may be put on the column; and any gel, no matter how adsorptive, may be used. The titrations, of which there are a large number, are carried out with KOH in methanol, which provides a monophasic system; this is a decided improvement.

Peterson & Johnson (1948) used Celite 545 as the carrier in place of silica gel, and, with water as the polar phase, showed that such columns could be used for the quantitative separation of the first four acids of the homologous series. The developing solvent for butyric and propionic acids was benzene; CB 10 was used to elute acetic acid, and CB 25 formic acid; butyric acid could not be separated from valeric acid. To separate the higher acids, the polar phase was changed to 28–36 N-sulphuric acid; the precise concentration used was conditioned by the nature of the acids to be separated. The developing solvents were thiophene-free benzene and thiophene-free benzene containing 2 % (v/v) Skellysolve B (?). Unfortunately, no data are presented on the behaviour on the column of those acids which contain an odd number of carbon atoms (propionic and formic excepted), and thus it is impossible to compare it with the method of Moyle *et al.* (1948), who have used a far more rigorous test; a false impression of efficiency is readily obtained by separating alternate members of an homologous series. A column with 30·5 N-H_2SO_4 as the polar phase was used to separate hexanoic acid from octanoic acid; and a 33 N-H_2SO_4 column was used for the separation of octanoic from decanoic acid. Thus, by using a series of such columns, it was possible both to separate and estimate the lower fatty acids containing an even number of carbon atoms in cheese.

If these two procedures are compared I think the buffered column method proves to be superior on all counts; not least of its advantages is the freedom from the hazard of grinding the carrier with concentrated sulphuric acid. However, the fact that a new carrier has been discovered is an important advance, and I cannot help feeling that it might, with advantage, be substituted for silica gel in a number of methods.

It would appear from both of these investigations, and from the theoretical considerations made at the beginning, that the jump from butyric acid and valeric acid necessitates the loss of the very convenient indicator technique. With further investigations of solvent

systems this loss need not be permanent (cf. Ramsey & Patterson, 1948). The loss of the indicator technique results in a great increase in the amount of work, for, whereas in the simple method the estimation of each acid required but one titration, five, ten or more titrations are needed when the columns are run blind. Nor are external indicators or electrometric devices likely to be of much use in these methods, since the bulk of the acids are in the organic phase; this, after all, is the reason why the immobile phase has to be altered in the first place. A saving might be effected by combining the original method with the buffered column method. A bromo-cresol green column would replace column I; the butyric acid fraction, along with the higher acids, could be quantitatively transferred to column II and further resolved (this is possible because buffered columns seem able to handle larger volumes than simple columns), and the propionic and acetic acids estimated as usual.

Recently, a new approach to the problem has been made. It is obvious that, could the non-polar phase be made immobile, and the polar phase used as the developing solvent, then the position would be reversed; i.e. the lower fatty acids would move rapidly and the higher acids slowly. This has now been translated into practice by Boldingh (1948). Filter-paper was impregnated with dilute rubber latex in such a way that it retained its porosity and yet contained 30% (w/w) rubber. Using methanol as the mobile phase it was possible to separate the ethyl esters of stearic, palmitic, myristic and lauric acids; with 1 : 1 methanol and acetone as the developing solvent, the esters of stearic, palmitic, oleic and erucic acids were separated.

To sum up, partition chromatography enables us to identify, separate, and estimate quantitatively the so-called volatile fatty acids. For tracer work the method will be invaluable, as it is vital to have the compounds in a state of purity before they can be assayed; prior to the development of the partition chromatogram it was impossible to separate the individual fatty acids in a mixture. The advent of the method also means that a study of the metabolism of these compounds is a practical possibility.

THE DI- AND TRICARBOXYLIC ACIDS

The individual members of this group of compounds are more amenable to chemical analysis than are the lower fatty acids, as each has characteristic chemical properties, but their quantitative separation from mixtures has always been difficult and, indeed, seldom possible on a micro-scale. Partition chromatography has made such separations possible, and there are now available two methods for the handling of mixtures of these compounds, one of them being quantitative.

Isherwood (1946) has applied the silica gel column to the analysis of mixtures containing fumaric, succinic, malic, oxalic, citric and tartaric acids. Two difficulties had to be overcome: first of all the acids tailed badly because of ionization, and secondly, silica gel, as usually prepared, adsorbed these acids, particularly oxalic acid. The problem of ionization was solved by the use of 0·5N-H_2SO_4 as the polar phase; ionization was thereby suppressed and symmetrical bands obtained. The adsorptive properties of the gel were removed by modifying the method of preparation. Adsorption was due in part to the presence of heavy metals, and these were removed by treating the gel with 10N-HCl. Two successive acid treatments, at intervals of 21 days during which period the gel 'aged', were necessary. This treatment produced gels which were non-adsorptive, at least towards oxalic acid; though it is interesting to note that Tristram (1946) reports that for the separation of acetyl amino-acids Isherwood's gels were neither better nor worse than gels prepared by the usual method.

Naturally enough the incorporation of 0·5N-H_2SO_4 in the aqueous phase makes it impossible to use the indicator technique on the column; but even were there no need for the H_2SO_4, the fact that CB 35 has to be used to elute certain acids makes it more than likely that columns would still have to be run blind, for all indicators are likely to be leached by this solvent. Instead of taking fractions and titrating each separately, as in the methods already discussed, an external indicator was applied. Very small drops of an indicator solution (thymol blue was judged to be the most suitable), were allowed to mix with the developing solvent as it issued from the column and in this way it was found possible to follow the acid content of the solvent, and thus to make the cuts. The flow rate of the indicator was about 0·1 ml./min.

From Isherwood's results it would appear that this is a useful method to employ where blind columns have to be run. A small correction, approximately 2%, has to be applied because of the slight excess of alkali in the indicator solution; in addition, CB 35 removes traces of H_2SO_4 from the column, but these are more than counterbalanced by the excess alkali in the indicator. It seems a pity that Isherwood omitted to include a complete protocol in his paper, so that the importance of these corrections could be evaluated.

Lugg & Overell (1947) have adapted the paper-strip technique to the separation of a similar group of acids. The developing solvent was *n*-butanol, and ionization of the acids—and thus tailing—was suppressed by the use of formic acid. The advantage of formic acid is that it is volatile and is removed from the paper by drying in the oven. The location of the acids on the paper is achieved by spraying with an acid-base indicator, bromo-cresol green. During the drying process a certain

amount of esterification occurs and the method is not quantitative. Should it prove possible to inhibit esterification then a very important analytical tool will have been developed, the more so since this group of compounds are intermediary metabolites of increasing significance.

PURINES, PYRIMIDINES AND THEIR DERIVATIVES

The quantitative separation and estimation of this group of compounds has been as difficult as it is important, but it now appears that they can be handled by the partition chromatogram, and that this technique will be as useful in the analysis of nucleic acids as it has been in the analysis of proteins. During the past year a number of methods have been published, and of these the most adequately described is that of Hotchkiss (1948). He used paper chromatography, and with *n*-butanol as the developing solvent was able to separate the bases adenine, cytosine, thymine, and uracil (guanine did not move with this solvent system), the ribose nucleosides adenosine, guanosine and cytidine, and the desoxyribose derivatives of guanine and thymine. He was unable to demonstrate these compounds visually on the paper strip; so, at the end of development, and after the removal of the solvent, the strip was cut into small segments and the eluate from each examined in the Beckman spectrophotometer at a wavelength of 260 mμ. The position of each compound on the strip was thus ascertained. The fractions were identified by a more extensive spectroscopic examination, and the publication is enriched by the inclusion of the complete spectroscopic data for each of the compounds dealt with.

Vischer & Chargaff (1947, 1948), in two brief notes, have outlined paper-strip methods for this group of compounds. In their first method (Vischer & Chargaff, 1947) the developing solvent was a 3 : 1 mixture of quinoline and collidine, and the bases were made visible by converting them to their mercury derivatives, and, after removing the excess reagent (0·25 M-$Hg(NO_3)_2$ in 0·5 M-HNO_3), spraying with aqueous $(NH_4)_2S$, whereby the bases were located as black spots. The solvent system used was such as to preclude the estimation of the bases by ultra-violet spectrophotometry. In their second note (Vischer & Chargaff, 1948) they described a quantitative paper-strip method. Two solvent systems were used: a mixture of *n*-butanol, morpholine, diethylene glycol and water for the purines and *n*-butanol and water for the pyrimidines. No details were given of the method of estimation, but presumably it was spectrophotometric.

The fact that these compounds can be separated on paper strips suggests strongly that starch columns could be utilized for preparative work. Reichard (1948) has recently shown this to be so, and with a

butanol-water solvent system has separated the ribonucleosides, adenosine, cytidine, guanosine and uridine.

Crammer (1948) has studied the behaviour of flavin derivatives on paper strips and has been able to separate flavin-adenine dinucleotide, riboflavin phosphate and riboflavin, using either collidine or a mixture of *n*-butanol and acetic acid as the developing solvent. The position of the compounds was indicated by their fluorescence in ultra-violet light.

Much of this work is very recent and must be considered as preliminary; none the less it is quite clear that this new technique is going to have far-reaching effects, and not merely on the more structural side of biochemistry but also on metabolism. For not only does it provide a method of analysis but it also enables us to isolate, in a state of purity seldom possible before, metabolic intermediaries, and this will be of immense importance in tracer work. It has not been sufficiently emphasized in the past that a primary requisite of tracer work is that the compound to be assayed must be in a state of purity. This is particularly important where radioactive tracers of high specific activity are used, and where a little contamination may go a very long way—even into students' text-books!

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CONTRIBUTIONS TO THE DISCUSSION OF ABOVE PAPER

Contribution by Dr Lester Smith. *Glaxo Laboratories, Greenford, Middlesex*

Dr Lester Smith said that he had lately been attempting the separation of some higher fatty acids, arising from acid hydrolysis of the penicillins, by partition chromatography, with a view to using this technique for estimating the individual penicillins in a mixture. The most promising method seemed

to be one that had not been mentioned by Dr Elsden; it had been described by Ramsey & Patterson (1948) and involved the use of silica damped with methanol containing bromo-cresol green indicator, with *cyclo*hexane or *iso*-octane as mobile phase. It was easy to separate phenylacetic, hexanoic and octanoic acids, arising respectively from benzyl-, amyl- and heptyl-penicillin. So far, however, it had not proved possible to achieve complete recovery of phenylacetic acid, possibly owing to esterification with the methanol.

Dr Lester Smith also referred to his use of partition chromatography for purification of the anti-pernicious anaemia factor of liver, using mainly silica columns with *n*-butanol as mobile phase, as described at a recent meeting of the Biochemical Society (Lester Smith & Parker, 1948). This technique had been at once a dramatic success and a dramatic failure. Thus the first chromatogram of adequately purified liver concentrate showed a pink zone which was subsequently shown to be due to the active principle. On the other hand, after chromatographing this active fraction once or twice more, a product was obtained that appeared to be chromatographically homogeneous, but that in fact contained only about 3 % of the pure crystalline anti-pernicious anaemia factor, along with peptides that could only be separated chromatographically after enzymic proteolysis.

The use of starch as the base instead of silica gave no more effective purification, although it did permit the separation of two clinically active pink fractions, provided the columns were run slowly enough. The reason for the failure to achieve complete chromatographic purification did not appear to lie in conjugation of the active principle with a large peptide, because the molecular weight by the diffusion method was approximately the same before and after proteolysis.

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Contribution by Prof. T. A. Bennet-Clark

Bands run in blind columns are very easily located by the electro-conductivity of the eluate.

Contribution by Dr R. R. Goodall. *I.C.I. (Dyestuffs) Ltd., Blackley, Manchester*

Dr Goodall had found that the water-absorbing power of kieselguhr was considerably less than that of silica gel of corresponding particle dimensions, and hence the capacity of a kieselguhr column was less than that of a silica gel column. He called attention to a beaten cellulose of uniform fibre length (passing 150 B.S. sieve) marketed by Johnsen, Jörgensen and Wettre Ltd., 26 Farringdon Street, London, E.C. 4, from which excellent columns bearing about 30 % of water could be made. The cellulose columns might also have higher affinity for colorimetric indicators than silica columns.

8. PARTITION CHROMATOGRAPHY WITH STATIONARY PHASE OTHER THAN PURE WATER

By A. A. LEVI

Imperial Chemical Industries Ltd., Hexagon House, Manchester 9

Solvent extracts of acidified penicillin broth contain a complex mixture of acids. Up to 1941 existing chromatographic techniques were not suitable for analysing this type of mixture, because (*a*) acids were usually so strongly adsorbed as to be incapable of development, and (*b*) the alkalinity of many adsorbents appeared to cause substantial loss of biological activity. It was found that a partial separation of pigments from biological activity could be achieved by repeated partition of the solvent extracts with phosphate buffers in tap funnels (Levi & Terjesen, 1943). An attempt was made to use this fact in a 'spinner' type counter-current extractor (Schutze, Quebedeaux & Lochte, 1938). In spite of elaborate ice jacketing, however, it was impossible to avoid considerable loss of biological activity, although some purification was obtained, and also some evidence that there might be more than one 'penicillin'.

At about this time the first publication on partition chromatography appeared (Martin & Synge, 1941). It was apparent that penicillin acid was too unstable and probably too little soluble in water to permit the use of the technique in its original form.

When, however, phosphate buffer was substituted for water as the sorbed stationary phase, columns were obtained showing a large number of coloured and fluorescent bands, which moved down the column at different speeds on the addition of further solvent. The biological activity was mainly located after development just above a prominent orange band. The material in this band gave a red coloration with acetic acid containing concentrated sulphuric acid as described by Catch, Cook & Heilbron (1942). This material was biologically inactive. A weaker zone of activity was later located below the orange zone, and another near the top of the column. Some indication was also found that the main zone itself contained more than one active material. Finally, we received from Messrs Merck and Co. in America a sample of penicillin which appeared to have different properties from the partially purified material isolated in these laboratories. Samples of each were mixed and put on a buffered column with ether as flowing phase, when

the mixture was clearly resolved into two components (Fig. 1). This was the first unequivocal demonstration of the existence of more than one penicillin. The actual experiment was carried out by Dr C. T. Calam.

Columns of this type have been widely used in the penicillin field, and the extension to paper strips, again inspired by Dr Martin and his colleagues (Consden, Gordon & Martin, 1944) has already been adequately described (Goodall & Levi, 1946, 1947). So far, however, there has been

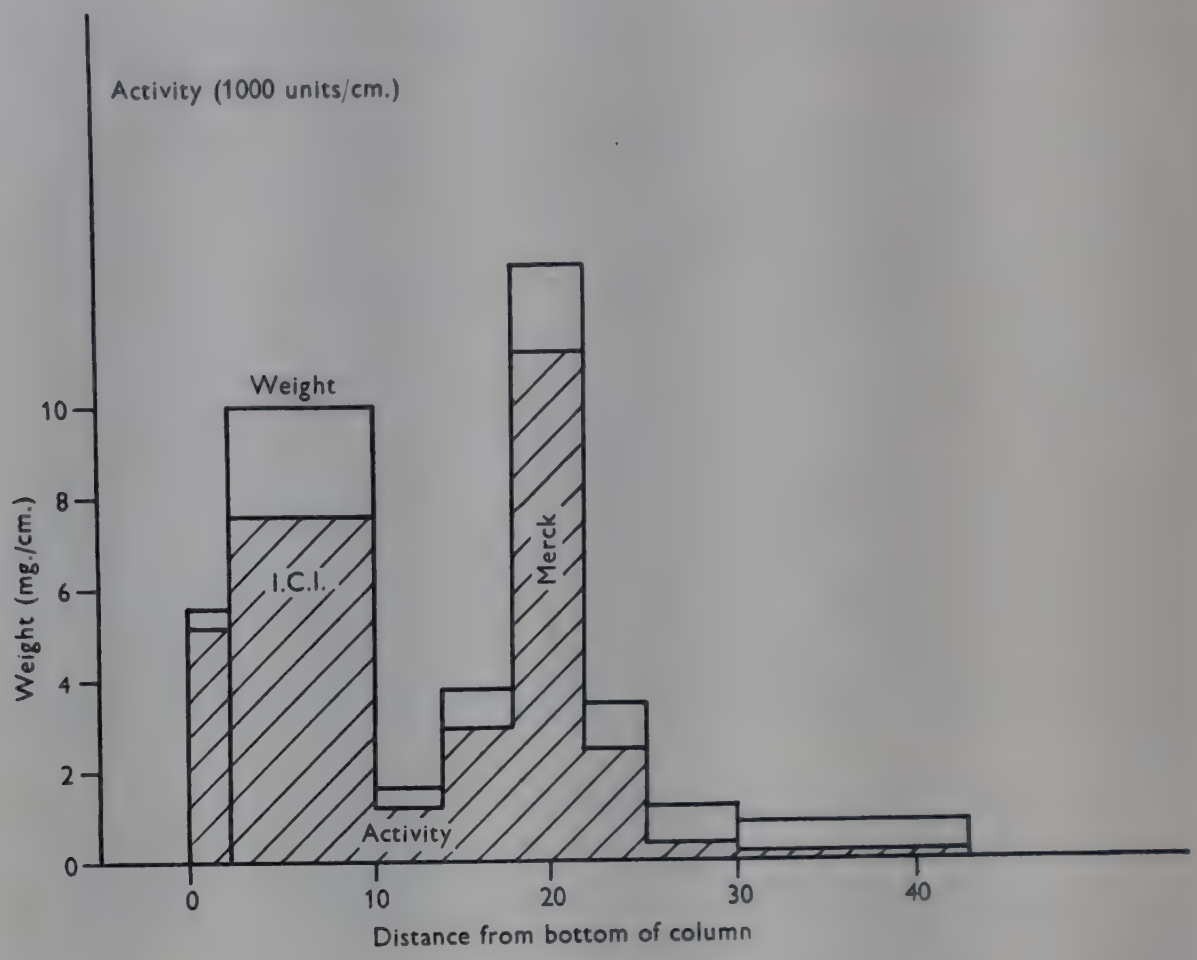

Fig. 1.

little opportunity to extend the method to other fields, and, so far as we are aware, few workers have published examples of the use of the technique for other separations.

The advantages of this type of column are:

(1) Acids which are more soluble in solvents than in water cannot give chromatograms by the original technique of Martin & Synge. The use of buffer solutions overcomes this difficulty, and since the pH of the buffer phase can be varied, the range of possible separations is widened.

(2) Extremes of acidity and alkalinity are avoided. This was particularly important for penicillin. No great loss of activity occurs when columns of this type are run at room temperature.

(3) By the use of concentrated buffer solutions relatively heavy loads

can be successfully separated. For example, columns consisting of 120 g. of silica and 60 ml. of strong potassium phosphate buffer solution gave substantial purification of the penicillin in 5 g. of crude material.

(4) The presence of trace impurities, e.g. alkali, iron, etc., in the silica is of much less importance than in columns containing only water as stationary phase.

The disadvantages are:

(1) Since indicators incorporated in the column are no longer usable it is necessary to devise some technique to detect the zones with colourless substances. In the case of the penicillins it was fortunately possible to use optical activity as a guide to the location of the important zones.

(2) For all substances so far examined the relation between partition ratio and concentration has been of the Freundlich isotherm type. This means that the bands invariably have tails, so that the complete separation of substances whose partition characteristics are rather similar may be difficult or even impossible except with very small quantities. Fig. 2 shows some isotherms drawn on a log scale for phenylacetic and benzoic acids. It is seen that good straight lines are obtained over a relatively wide range of concentrations. It is further seen that isotherms may have different slopes, a fact which may still further hinder separation in some cases.

In spite of these disadvantages the technique should be of value in a number of other fields. Thus the detection and isolation of minor alkaloids should be greatly facilitated with suitable columns. A few determinations of partition ratios in tap funnels will soon indicate the type of system likely to give useful separations. A recent publication by Evans & Partridge (1948) describes some successful examples of this application of the technique. The possibility was foreshadowed by Catch *et al.* (1942) in their letter to *Nature*, and in a patent (1943) which also describes a separation of acetic and pyruvic acids.

In these publications there was described another variant of the partition chromatogram. These authors prepared silica-gel columns which were impregnated with solutions of alkali, or alkaline earth hydroxides or carbonates.

When solvent solutions of, for example, crude penicillin are poured through such columns sharp banding is obtained, but the bands are incapable of development.

Theory shows that the distribution of the material in the zones must be similar to that obtained in 'frontal analysis' as described by Claesson (1946, 1947). That is, the lowest zone consists only of the most weakly acidic substance. The next zone consists of this zone plus the next most weakly acidic substance, the amounts of each depending mainly on their relative strengths as acids. The next zone

contains a third substance in competition with the two already present, and so on up the column. There is, however, this difference from frontal analysis in that all the zones will have the same total molecular concentration, which is controlled by that of the base in the stationary phase. It is clear that complete separation cannot be effected by this means. Nevertheless, the technique has been found valuable for a

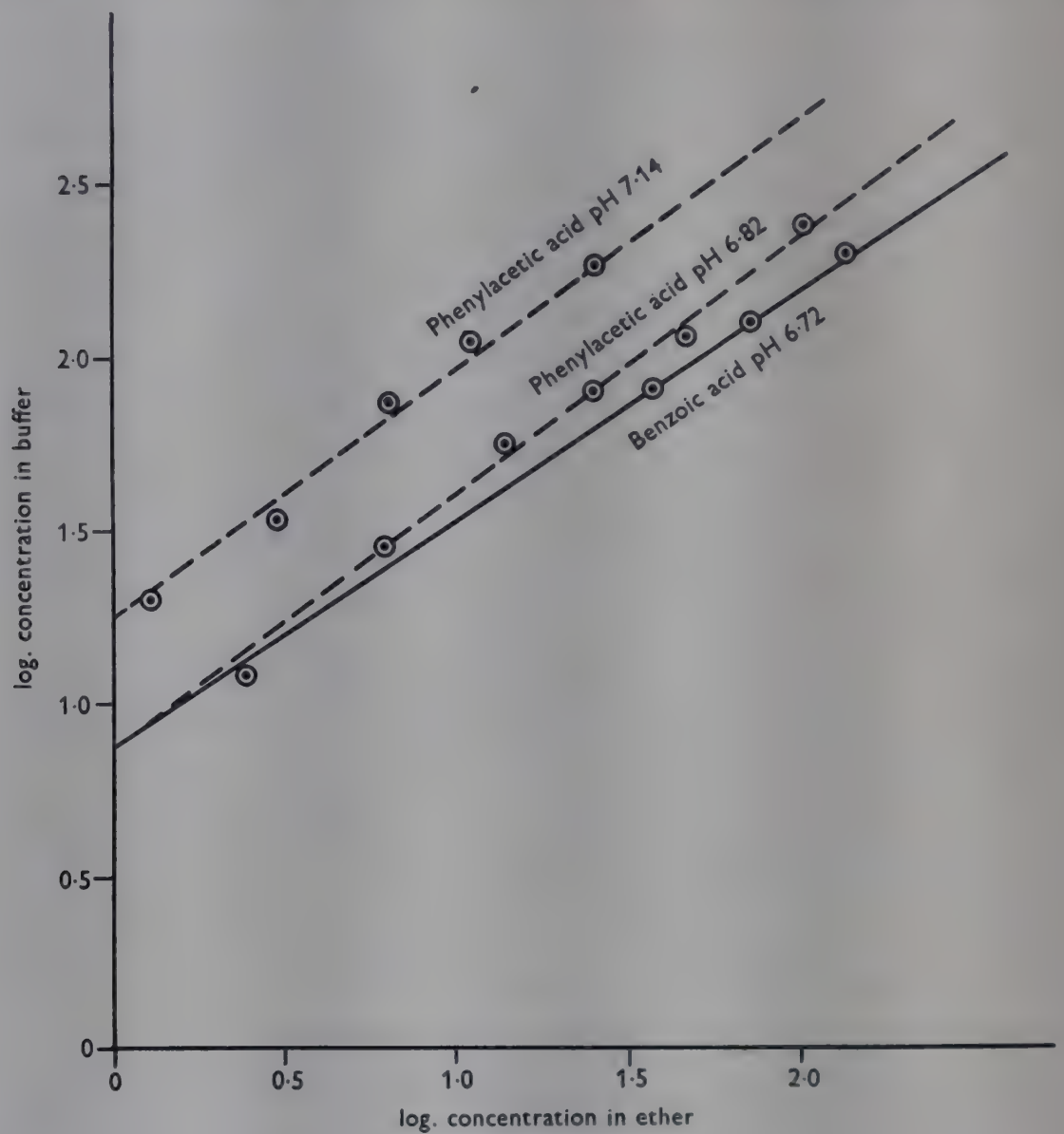

Fig. 2.

preliminary purification of very crude penicillin preparations. For this purpose we have used silica impregnated with caustic soda of up to 10% strength. By this means it was possible to effect substantial purification of quite large quantities of material on relatively small columns. That this is the case will be realized when it is remembered that a 2M solution of penicillin, mol.wt. about 300, will have a strength of approx. 60%. With 4N-sodium hydroxide the columns tended to disintegrate, no doubt due to the large changes in volume which occurred when the base was neutralized. That the distribution of the zones was substantially

as predicted by theory was shown by a series of paper-strip chromatograms on fractions cut from the column. There was, however, one complication not provided for in the above theory. This was due to the fact that most of the acids in crude penicillin are more or less soluble in water. Hence after all the base on the column had been stoichiometrically neutralized more acidic substance dissolved in the aqueous phase due to water-solvent partition alone. This was shown to be the case by the fact that the pH of the material extracted from the separated zones decreased from about 5 at the bottom to about 3 at the top of the column. This had the undesirable effect of causing substantial inactivation of the penicillin unless the columns were run in a cold room. There is little doubt that the original columns for penicillin described by Abraham, Chain & Holiday (1942), using ordinary chromatographic alumina, behaved in much the same fashion as these caustic soda columns due to the alkalinity of the alumina, and in the same way substantial inactivation occurred on these columns unless they were refrigerated. This inactivation was probably not due to excessive alkalinity, as is usually supposed, but to excessive acidity. In spite of this difficulty, the technique gave a simple and invaluable preliminary purification, although its use has now been superseded by better methods.

It is obvious that a crude solvent extract of bases could be similarly fractionated using, say, hydrochloric or sulphuric acid on the column.

Dilute sulphuric acid has already been used on columns by Isherwood (1946), but the purpose here was simply to reduce the ionization of simple organic acids so as to give more nearly linear isotherms.

There must be other reversible and irreversible systems in which a solution of some substance held on the silica has different affinities for the components of a mixture dissolved in a suitable non-miscible moving phase.

It seems worth while pointing out here that there is another method for dealing with substances which show bad 'tailing', namely, the Tiselius method of displacement development (Tiselius, 1943). In this method of chromatography the column is prepared and the charge put on in the usual way. A developer is now chosen which has a higher distribution ratio in favour of the stationary phase than any component of the mixture. A solution of this substance is now poured on to the column, when the mixture resolves itself into a series of contiguous bands, each containing a pure single component. With a given developer the concentration in each zone is characteristic for the particular component, and can be used for its identification. The width of the zone, or the volume required to move it past a given point, multiplied by this characteristic concentration, gives the weight of

solute in the zone. Under suitable conditions, therefore, a complete qualitative and quantitative analysis is possible on a single column. The point to be stressed is that the success of the method depends on the existence of the very factors leading to tailing on an ordinary chromatogram. In these laboratories Dr Goodall has succeeded in applying the method on a partition chromatogram for the first time to such a system, the results for which it is hoped to publish in the near future. It is only fair to add, however, that the experimental difficulties have been considerable.

Finally, a few attempts have been made to use non-aqueous phases on the silica gel. In an early experiment with penicillin, silica gel was impregnated with amyl acetate, and a solution of crude penicillin in phosphate buffer was passed through the column. Banding was certainly visible, but was very diffuse. A better method is possibly that outlined by Boscott (1947), in which the stationary phase consists of cellulose acetate swelled with butanol or other solvents.

More success has attended the use of adsorbed nitromethane with the mixture to be analysed dissolved in methanol. Ramsay & Patterson (1945) have analysed hexachloro*cyclo*hexane very satisfactorily by this means.

The same authors have this year described two other such systems, which have been applied to the separation of the lower and higher fatty acids respectively (Ramsay & Patterson, 1948). The first system, for acids from C_5 to C_{10}, consists of adsorbed methanol with *iso*octane as the flowing phase. Methanol and petrol ether have of course frequently been used in tap funnels in the past. This system should have some flexibility on partition chromatograms, since partition ratios should be variable by variation of the water content of the methanol.

The second system, for acids from C_{11} upwards, consists of equal volumes of furfuryl alcohol and 2-aminopyridine on the silica, with *n*-hexane or *iso*octane as flowing phase. Mixtures of acids with all odd or all even numbers of carbon atoms were successfully separated, but separation was incomplete with mixtures of odd and even numbers of carbon atoms.

It will be seen from this brief survey that there is quite a large field open for various types of chromatographic separations using partition columns with stationary phases other than water alone.

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CONTRIBUTION TO THE DISCUSSION OF ABOVE PAPER

Contribution by Dr Lester Smith. *Glaxo Laboratories, Greenford, Middlesex*

Dr Lester Smith said that he had prepared penicillin labelled with radioactive sulphur for another purpose (Rowley, Miller, Rowlands & Lester Smith, 1948; Rowlands, Rowley & Stewart, 1948), and it seemed of interest to utilize its radioactivity to determine the proportions of the individual penicillins present. Micro-drops, containing about 5 units, were applied to buffered filter-paper strips, which were developed with ether after the method of Goodall & Levi (1947). The penicillin spots were located by making a radio-autograph from one of the strips.

With this as a guide, the paper was cut into squares in such a way as to keep the individual penicillins separate. The β-radiation from successive sections of the strip was then measured with a Geiger-Müller counter having a thin mica end-window.

A number of other strips were applied to agar plates seeded with *B. subtilis*, and the proportions of the penicillins calculated from the diameters of the zones of inhibition.

The results by the two methods agreed well.

Penicillin type	% by weight	
	Geiger-Müller	Microbiological method
G	84·5	87
F	8·5	6·5
Dihydro F	2	1
K	5	5·5

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9. GENERAL REVIEW OF THE APPLICABILITY OF THE METHOD

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It is indeed gratifying to note how, as set forth in the present Symposium, the technique of partition chromatography has already found application for effecting separations within nearly all the families of low-molecular organic substances with which biochemists are concerned. To the groups dealt with here must be added a wide range of inorganic elements, many of which are of great concern for biochemists. Linstead and his colleagues at Teddington have shown these to be separable in microgram quantities by methods essentially those of partition chromatography (Arden, Burstall, Davies, Lewis & Linstead, 1948; cf. Lederer, 1948; Stene, 1944; Burstall, Davies, Linstead & Wells, 1949).

Although still in the early stages of its application to biological systems, partition chromatography has done much to reveal the complex array of smaller molecules occurring in them, and has also permitted a few simplifying generalizations. Experience has also been gained in extending partition chromatography and other new purification techniques towards separations of rather larger molecules (polypeptides are a good example). This should help to develop healthy scepticism as to the validity of the criteria of purity that are still widely accepted for really large molecules of biological origin.

The advent of partition chromatography has greatly stimulated two developments, neither of which is essentially new to the chromatographic art. I refer to the use of paper and to the application of chemical equilibrium reactions in addition to 'purely physical' or 'secondary valence' or 'molecular' interactions.

Filter-paper chromatography, using what Tiselius has taught us to call 'front analysis', was applied as early as 1861 by Schönbein, long before Tsvet's great discovery of the use of adsorbent columns with 'elution development' accompanied by more or less of 'displacement development'. Schönbein's pupil Goppelsroeder (1901) published an extensive and incoherent book on his filter-paper work, which reveals far less theoretical understanding than the writings of Tsvet. Nevertheless, filter-paper chromatography has found continuous application in the analytical study of dyes and related substances ever since. With the advent of partition chromatography, better development procedures

have been evolved, notably the 'two-dimensional' procedure (Consden, Gordon & Martin, 1944), and fuller advantage has been taken of the coherence and manipulability of paper in order to detect ultra-micro quantities of materials. For this purpose there are available a wide variety of spot reagents applied with a spray; cutting out for subsequent testing is simpler than with powder columns; radio-autographic contact-printing on to a photographic plate may be used where radioactive substances are present (Fink, Dent & Fink, 1947)*; and substances on paper chromatograms may be 'printed' on to solid culture media for subsequent testing of biological activity with micro-organisms (Goodall & Levi, 1946).

Chemical equilibria such as ionization, salt formation and the reversible formation of complexes and derivatives can profoundly influence the distribution of substances between the mobile and stationary phases of chromatograms and thus be of value for effecting separations. In adsorption and ion-exchange chromatography, reagents controlling such distributions can easily be added to the mobile phase (e.g. the use of formaldehyde for differentiating the behaviour of amino-acids and peptides on alumina chromatograms (Jutisz & Lederer, 1947)). However, it is not such a simple matter to concentrate chemical reagents at will on the surface of solid adsorbents. Synthetic polymers are generally most promising for this purpose, and they have already played an important role in the provision of surfaces with ion-exchanging groups having the required dissociation constants. The non-adsorbed groups of strongly adsorbed molecules may also be utilized, as exemplified by ion-exchanging properties of charcoal treated with aromatic acids and bases (Steenberg, 1944). In partition chromatography, chemical equilibria in the stationary phase may occur under the better understood conditions prevailing in a bulk phase. Indeed, the first partition chromatogram described (Martin & Synge, 1941*b*) utilized such an equilibrium reaction—that of an acid-base indicator—although for observational purposes only. Dr Levi has described the utilization of buffers, acids and bases for controlling ionization equilibria in partition chromatograms, and he and Dr Martin in their respective contributions have dealt with the application of displacement development methods to such systems. We can hope shortly to hear of modification of partition coefficients of solutes by salt formation with added organic acids or bases, to increase their solubility in non-polar solvents. Many of the achievements of Linstead and colleagues in the inorganic field clearly depend on equilibria involving complex formation by the metals. Dr Dent has mentioned a converse process, utilizing complex formation with copper (cf. Consden *et al.* 1944; Moore & Stein, 1948), to separate

* (Note added in proof.) See especially the beautiful metabolic study of photosynthetic reactions by Calvin & Benson (1949).

α- from β-, γ-, etc. amino-acids. The use of chelation of organic acids with other organic acids in non-polar solvents has been described by Dr Martin, and probably also plays a role in improving the linearity of the partition isotherm in Lugg & Overell's (1947) chromatograms with fruit acids. The use of bisulphite has been suggested for controlling the behaviour of aldehydes and sugars. Dr Martin has even dared to suggest the use of more slowly attained equilibria, such as the esterification reactions of organic acids and alcohols. One may further hope to utilize enzymes for specific separations of their 'competitive inhibitors' where the Michaelis constants differ suitably, and similarly antibodies for separations of haptens. No descriptions seem yet to have been published of isotope separations in partition chromatograms by utilizing exchange equilibria, as originally suggested by Martin & Synge (1941*b*), but there seems no reason to doubt that such separations can be facilitated by the very high resolving power of the chromatographic arrangement. There seem, in fact, to be no limits in sight to the extension of what Catch, Cook & Heilbron (1942) have called 'chemical chromatography', and the conditions prevailing in partition chromatograms are a great aid in realizing such systems.

Now consider the *essential* features of partition chromatography. It is convenient to treat these in relation to the techniques of counter-current liquid-liquid extraction and adsorption chromatography. This emphasizes the theoretical similarity of the methods, already stressed by Dr Martin in his contribution, and helps to make clear the particular advantages of each in relation to different problems.

Partition chromatography originated in a very direct way from a consideration of counter-current liquid-liquid extraction trains. At the period in question, only continuously operating trains had been applied for biochemical purposes (Cornish, Archibald, Murphy & Evans, 1934; Martin, 1937; Martin & Synge, 1941*a*). In recent years it has become recognized (e.g. Craig, 1944; Stene, 1944; Bush & Densen, 1948) that discontinuous operation of the train (that is, to separate the operations of mixing, settling and transfer in time rather than in space) has for some purposes both theoretical and practical advantages. In the hands of Craig such discontinuously operated bulk liquid-liquid extraction trains have been shown to have a wide applicability in biochemistry. It is of interest that Stene (1944), from a study of theoretical problems of this kind, was led independently to try to realize a partition chromatogram at almost exactly the same time as Martin and I were working at the problem. The system tried by him consisted of droplets of toluene immobilized in an aqueous agar jelly. Difficulties seem to have been encountered in moving the water relative to the toluene without disrupting the jelly.

The use of bulk liquid-liquid extraction trains (whether continuously or discontinuously operated) means that the large number of theoretical plates, i.e. resolving power, that are embodied in even quite a short chromatogram, cannot be realized without excessively bulky apparatus. Moreover, chromatograms are usually much simpler to manipulate.

On the other hand, in partition chromatograms, it is often difficult in practice to take advantage of the large number of theoretical plates available. Under these and other circumstances extraction trains may have special advantages. Thus when adsorption on the supporting substance of the chromatogram is appreciable, and conforms to a very curved isotherm, the extraction train may permit easier realization of a system conforming to Henry's law. (Here it should be pointed out that continuously operated extraction trains may permanently contain a large area of liquid-liquid interface at which adsorption may occur and interfere with separations. Behaviour of this type was observed with certain sulphur compounds in the chloroform-water train of Martin & Synge (1941*a*). Discontinuous operation minimizes this source of trouble.)

There is also presumably an upper limit of molecular weight for solutes on partition chromatograms, above which they cannot penetrate the pores of the supporting substance. No such limit exists for bulk liquid-liquid extraction. The use of kieselguhr, described by Dr Martin in his contribution, may get over this difficulty and help generally towards the realization of an 'ideal' partition chromatogram.

The partition chromatogram cannot take full advantage of proportional differences in partition coefficients when these are in the range leading to R or R_F values greater than 0·3–0·5. Where partition coefficients are greatly in favour of the moving phase, useful chromatograms should be obtainable by holding the other phase in the supporting substance. Nearly all supporting substances so far used hold the more polar phase preferentially, but attempts have been made to reverse this, using cellulose acetate (Boscott, 1947) and rubber-impregnated paper (Boldingh, 1948). Boldingh describes actual separations of esters of higher fatty acids, and there is clearly a promising field of application here among lipoids, sterols, etc.

Another way of dealing with partition coefficients unduly in favour of the moving, less polar phase is of course to add a fresh solvent that will modify the partition coefficients in favour of the more polar phase. Where this involves the addition of such non-polar solvents as petroleum, carbon tetrachloride, etc., adsorption effects on the columns may become tiresome.

Liquid-liquid extraction trains can, by contrast, be designed to work satisfactorily at a wide range of partition coefficients, and the so-called

'Craig machine' works best with partition coefficients near unity, which is a difficult range for partition chromatography. Finally, within convenient laboratory dimensions, extraction trains handle greater absolute quantities of solutes than do partition chromatograms.

Dr Martin has analysed and compared in some detail the physical forces determining the distribution isotherms for solutes between two liquid phases and between a liquid phase and a solid surface, and has shown how, in a very general way, partition chromatograms are better for separating successive members of a homologous series and adsorption chromatograms for separating stereoisomers. The use of mixed solvents in partition chromatography clearly sometimes permits a subtle balancing of intermolecular forces, with one solvent associated more with a particular part of the solute molecule, and another with another. This sort of mechanism may be responsible for the system butanol-benzyl alcohol-water being better for resolving leucine and isoleucine than either butanol-water or benzyl alcohol-water (cf. Consden *et al.* 1944; Moore & Stein, 1948). However, such cases do not seem to be very common in partition chromatography, and it can show nothing to emulate the interplay of molecular forces in adsorption at solid surfaces, which permits quite small additions of new substances to the system profoundly to influence the distribution of a solute. This effect (which is usually, though not always, to diminish adsorption) must depend on realization of a high concentration at the adsorptive surface, their effects on the activity of the solute in the liquid phase being minor. Thus adsorption chromatography offers scope for the use of elution and displacement procedures without gross changes of solvent system that are forms of 'displacement development'. These are not available in partition chromatography until chemical reactions are called into play.

Of course adsorption and liquid-liquid partition effects are often present in one and the same chromatogram. That must be the case in many of the chromatographic systems described in this Symposium. In practice, adsorption effects may not hinder, or even may help separation, provided that the isotherms concerned are fairly linear *and* that the faster-moving component is also the less adsorbed. In a sense it is pedantic to argue about the relative roles of adsorption and partition in a chromatogram; the movement of a substance is determined by its distribution isotherm between mobile and stationary phases, to whatever set of mechanisms this is due. In passing, it is this fact that tells strongest against the view (e.g. Wachtel & Cassidy, 1943) that partition chromatography should not be referred to as chromatography. Surely the essential feature of chromatography is the mechanical arrangement for fluid percolation, with its very high plate number and resolving power. This feature seems to have been appreciated by

Tsvet; very weighty reasons would be needed for breaking away from a name so firmly rooted in history and sentiment.

There is not scope here for dealing with the physico-chemical distinction between 'adsorption' and 'partition' mechanisms in silica gel, starch grains, cellulose, etc., and in any case much further study of these substances and their equilibria with solvent mixtures is required. I think, though, that a broad distinction can be drawn between 'normal' adsorption effects at micellar surfaces within these substances, and effects due to modification of the composition and properties of the sorbed liquid phase. One would, in other words, be inclined to classify effects according to the spatial distribution, on an ultra-microscopic scale, of the solute molecules within the grain. Thinking on these lines, I would be inclined to agree with Moore & Stein (1948) that the deviations of aromatic amino-acids from 'ideal' behaviour on starch-butanol-water columns are due to adsorption effects, but would not hold that starch chromatograms cannot be partition chromatograms because they work well with solvents such as wet propanol that will not form two phases with water. The propanol-water ratio is not necessarily the same within the starch grain as outside it. In this connexion, failure so far to separate optical antipodes under partition chromatographic conditions on such asymmetric materials as cellulose and starch may be significant.

In conclusion, I would like to thank all who have contributed to the work of exposition and discussion embodied in this Symposium. I hope not only that this interchange of ideas will help us to improve the purely technical analytical work that most of us as biochemists have to carry out, but also that we shall learn, from study of the intermolecular forces between smaller molecules that are so strikingly revealed in chromatographic operations, to build up a systematic theoretical treatment that will help to interpret the highly specific interactions of larger molecules. We all know that an increased understanding of these interactions will be the key to great advances in our science.

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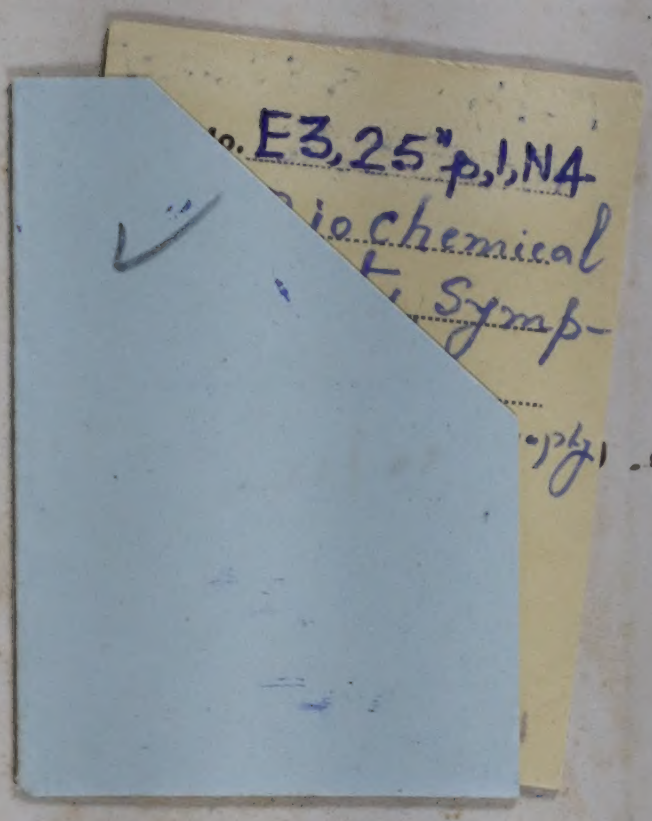
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